Dill University

College of agriculture and Natural resource

Department of Horticulture

Course Title; Introduction plant Biotechnology

Credit hours: 2(1+1)

Academic year: 2019/20, semester I

Program: year II Horticulture

Course instructors: Mitiku M.

1. Course description

The course covers: Introduction, plant tissue culture including media preparation, Initiation and maintenance of cultures organogenesis; somatic embryogenesis, micro-propagation, protoplast isolation, culture and fusion; Anther/pollen culture; somaclonal variation; germplasm conservation. Isozyme markers, DNA markers (RFLP, RAPD, AFLP, SSR, SNPs); applications of molecular markers; marker-assisted selection (MAS); mapping of genes and QTLs, Gene pyramiding. Gene manipulation; restriction endonucleases, ligases and other enzymes; cloning and expression vectors; Gene transfer methods, preparation and screening of genomic and cDNA libraries; DNA sequencing; Polymerase chain reaction: antisense RNA; ribozymes; gene mapping techniques; potential application of genetic engineering in agriculture; the impact of recombinant DNA technology; biosafety aspects & patents

2. Course objectives

At the end of the course units, students will be able to:

- Explain tissue and organ culture and its Application in agriculture
- Understand the basic requirements and techniques of plant tissue culture
- Differentiate the different molecular marker and their application in plant breeding.
- Understand the concept of genetic engineering
- Understand the potential of plant biotechnology (plant tissue culture, molecular marker, and genetic engineering) over the conventional plant propagation and breeding techniques.
- Summary concept of Plant biotechnology

3. Course Content

Reference

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Razdan M.K. and FSCD PH.D. 2002. Introduction to Plant Tissue culture, 2nd edit. Co.Pvt.Ltd, New Delhi.

Govi J.N, Kumar P.A and Singh ViK. 2004. Recent progress in Medicinal Plants; Biotechnology and Genetic engineerings. Studium press.llc, Texass, USA.

Satyanarayana U. 2005. Biotechnology.

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Purohit. S.S. 2007. Biotechnology fundamentals and applications

https://www.sciencedaily.com/news/plants_animals/biotechnology/

1. Total Assessment Methods

Assessment methods	**Allotted marks in %**
First assessment	10
Second assessment	15
Third assessment	10
Lab/Practical assessment	15
Final assessment	50
Total	**100%**
Grading: Fixed scale	

1. Introduction

What you expect from this course, do you know the current technology around the globe. Can you mention three current and more advanced technology globally? From your locality do you know any practices (medicinally, food related, culturally, etc.) related to living organism or part of them. How local food like injera, kocho, breads, etc and local beverages like tela, Areke, borde, tej etc prepared? Is there any involvement of living organism? Discuss with our friend come up with the answer.

What Is Biotechnology?

Definition

- ☞ Any technique that uses living organisms or their component(cell, tissue, etc)
  - ⇨ to make or modify a product,
  - ⇨ to improve plants or animals, or
  - ⇨ to develop microorganisms for specific uses.

- ☞ The **controlled use of biological agents** for beneficial use, such as
  - ⇨ microorganisms or cellular components,
  - ⇨ systems or processes,
  - ⇨ cells and tissues from higher organisms(plant or animal)
- ☞ The application of scientific and engineering principles
  - to the processing of materials by biological-agents
  - to provide goods and services
    - ⇨ It is the use microbial, animal or plant cells or enzymes to synthesize, breakdown or transform materials

Contrary to its name, biotechnology is not a single technology. Rather it is a group of technologies that share two (common) characteristics -- **working with living cells and their molecules** and having a wide range of practice uses that can improve our lives

It require **Knowledge**, **Principles**, **Science**, **Techniques**, **Technology**, **Application**, **Process** and **Industry**

1.1. Stages of Biotechnology Development

- ☞ For thousands of years, humankind has used biotechnology in agriculture, food production and medicine.
- ☞ The term itself is largely believed to have been coined in 1919 by Hungarian engineer **Karl Ereky**.
- ☞ In the late 20th and early 21st century, biotechnology has expanded
  - ⇨ to include new and diverse sciences such as
    - genomics, recombinant gene technologies, applied immunology, and
    - Development of pharmaceutical therapies and diagnostic tests.

1. Ancient biotechnology

- biotechnology has been practices since the beginning of records history
- early history as related to food and shelter; Includes **domestication**
  - ✓ domestication of animals,
  - ✓ cultivation of plants,

2. Classical biotechnology

- Also called Traditional/old biotechnology
- built on ancient biotechnology
- The conventional techniques that have been used to produce beer, wine, cheese, many other food and medicine
  - ✍ Anti-biotech production
  - ✍ Bioprocessing like **fermentation** using microbes
    - Baking -Making leavened bread – 4000 BC (Egyptians)
    - Brewing -Making beer – 6000 BC (Babylonians)
    - 1857 and 1876– the fermentative ability of microorganisms by Pasteur
    - **breedin**g programs that employ artificial selection and hybridization for "improvements"

3. Modern/new biotechnology

- ✍ All methods of genetic modification/ manipulates genetic information in organism
  - ⇨ Genetic engineering
  - ⇨ recombinant DNA and cell fusion techniques, together with the modern development of traditional biotechnological process
  - ⇨ Cell culture
  - ⇨ Fusion
  - ⇨ Bio-processing at molecular level

1.2. Areas of Biotechnology

It can be classified depending on different criteria, to discuss in detail is above the scope of this course. But the general over view is discuss as follow

Depending on the level of the study it can be classified as;

1. Organismic biotechnology

- ✍ uses intact organisms; Does not alter genetic material
  - molecular breeding
  - tissue culture

2. Molecular biotechnology

- ✍ alters genetic makeup to achieve specific goals
  - Transgenic organism- an organism with artificially altered genetic material

Depending on the field of study area it can also be classified as;

- ☞ Agricultural Biotechnology (Green Biotech)
- ☞ Food Biotechnology (Yellow Biotech)
- ☞ Pharmaceutical Biotechnology (Red Biotech)
- ☞ Industrial Biotechnology (White Biotech)
- ☞ Marine Biotechnology (Blue Biotech)

- ☞ Environmental Biotechnology (White Biotech)
- ☞ Biotransformations, Genetic Engineering, Biosensing & Nanotechnology

1. Medical Biotechnology

- ✍ Therapeutic products
- ✍ Vaccines production
- ✍ Immunodiagnostic and DNA probes for disease identification
- ✍ Pre- natal diagnosis of genetic diseases
- ✍ Gene therapy

2. Environmental Biotechnology

- ✍ Cleaning through **bioremediation**
- ✍ Preventing environmental problems
- Pollution presentation
- ✍ Monitoring the environment

3. **Agricultural Biotechnology**

- ✍ Plant biotechnology
- ✍ Animal biotechnology
- ✍ Food processing

If you apply biotechnology techniques on your surrounding you can achieve both clean environment and good business. How?

Plant Biotechnology

Can you mention technology behind plants?

Importance

- ✍ Crop production and protection
  - ⇨ Genetically engineered (transgenic) crops
  - ⇨ Using biological methods to protect crops
  - ⇨ Exploiting cooperative relationships in nature
- ✍ Nutritional value of crops
  - ⇨ Improving food quality and safety
  - ⇨ Healthier cooking oils by decreasing the concentration of saturated fatty acids in vegetable oils
  - ⇨ Functional foods
  - ⇨ Foods containing significant levels of biologically active components that impart health benefits

Classification

1. **Traditional plant biotechnology**
   - breeding
   - tissue culture
   - inter-specific hybridisation
   - mapping phenotypic/biochemical markers
2. **Modern Plant biotechnology**

   Now use a molecular approach to manipulation:
   - molecular markers & mapping
   - gene cloning
   - plant transformation

Inter-displinery course

- Cell biology
- Chemical engineering
- Computer Science
- Materials Science
- Genetics
- Immunology
- biochemistry
- Physiology
- Molecular biology
- Microbiology
- etc

1.3. Biotechnology in Ethiopia

What are the importance of biotechnology for Ethiopian development? In the second GDP the country gives great focus for biotechnology why? Which area of biotechnology more applicable to current status of the country and for the future? Our country is one of biodiversity reach area of the world, but utilization of this resource is very little. Do you believe that application biotechnological techniques improve this problem? How?

Ethiopia is an agrarian country that can have enormous benefit from the applications of biotechnology for increasing its agricultural productivity. The country is at initial stages of research and development in agricultural biotechnology with scattered efforts underway in various public institutions.

Research efforts and applications in crop production include

- plant tissue culture,
- bio fertilizers and bio pesticides,
- molecular markers for disease diagnosis
- Genetic diversity.

Livestock related applications include

- artificial insemination,
- molecular diagnostics,
- vaccine production and
- molecular genetic analysis.

The modern biotechnology is at its enfant age

Genetic engineering/modification

- It allowed only for some crops like nonfood crops otherwise it is restricted
- The risk taker is the research
- Infrastructure and skills in recombinant DNA and other cutting edge technologies such as proteomics and bioinformatics are still limited and need to be strengthened.

Ethiopia has recently given a due emphasis for

- National Agricultural Biotechnology Research Center (2010)(at Holeta): the Center conducts research activities at four of its laboratories in areas of
  - (1) plant tissue culture biotechnology research,

- (2) central molecular biotechnology research,
- (3) animal biotechnology research, and
- (4) microbial biotechnology research.

✍ A.A. University recently established the Institute of Biotechnology (June 2012)
✍ Last year(2008 E.C) general Ethiopia biotechnology road map is developed
⇨ The government of the country give attention
⇨ Different biotechnology center were established at different part of the country.
⇨ capacity building in agricultural biotechnology extending from promoting research, development and education
✍ in various public institutions to setting up of an independent agricultural biotechnology research center.
✍ Seven institutions with many branches are engaged in biotechnology research/teaching

1. Ethiopia biotechnology research center(holeta)
2. Addis Ababa University (AAU)
3. The Institute of Biodiversity Conservation and Research (IBCR).
4. Ethiopian Health and Nutritional Research Institute (EHNRI)
5. Ethiopian Agricultural Research Organization (EARO)(Melkassa)
6. National Veterinary Institute (NVI),
7. Institute of Pathobiology (IPB).
8. National Veterinary Institute at Debre Zeit

Higher education

- BSc- at Gonder, wolkite, etc
- Master program- A.A., Jimma, Heromay, Ariba minich, Dilla, Hawassa
- PHD -at A.A and Hawassa.

The current areas of emphasis biotechnology in Ethiopia are

✍ Agricultural Biotechnology
✍ Industrial Biotechnology
✍ Environmental Biotechnology

Agricultural Biotechnology

Plant Tissue Culture

❖ Very active research area
❖ Tissue culture protocols developed & optimized for
- Anchote (Coccinia abyssinica),
- Oromo or Woliyat Dinich (Plectranthus edulis),
- Tef

❖ Medicinal plant
- Metere (Glinus lotoides)
- Dingetegn (Taverniera abyssinica)

Breeding & Plant Genetic Resources

❖ Sorghum, Finger millet, Emmer wheat, Coffee, Lepidium sativum, Fenugreek, Enset

Plant Transformation

❖ Have the expertise and capacity
❖ Working towards establishing the facility

1.4. Achievements in biotechnology

Traditional

- Processing of food using microorganisms
  - Bear, areke, tela, ejijira using yeast
- Plant and animal breeding(hybridization)
  - Repeated cycles of selective breeding produced many present-day food staples

Modern

- the transferal of a specific gene from one organism to another
- the maintenance and growth of genetically uniform plant- and animal- cell cultures, called clones;
- the fusing of different types of cells to produce beneficial medical products such as monoclonal antibodies, which are designed to attack a specific type of foreign substance insulin and interferon

Application

- contributes to such diverse areas as food production, waste disposal, mining, and medicine.

2. Plant tissue culture

What is Plant Tissue Culture?

☞ Broadly refers to technique of growing plant cells, tissues, organs, seeds or other plant parts in a sterile environment on a nutrient medium in the laboratory

How could possible to grow cell, tissue, organs and etc. of a given plant?

Basis for the cell culture

✍ Plant cells are totipotent

- Have the ability to develop into whole plants or plant organs *in vitro* when given the correct conditions
- Not all plant cells are totipotent. However, there are a sufficient number of totipotent cells in the plant (e.g. in the pith)
- Differentiated cells have to be dedifferentiated into callus and redifferentiated back to somatic embryo that will regenerate the entire plant

2.1. Requirements for tissue culture

1. Laboratory
2. Appropriate tissue
3. Growth medium
4. Aseptic (sterile) conditions
5. Growth regulators
6. Frequent subculturing

2.1.1. Plant Tissue Culture Laboratory

Due to its strict hygienic requirement, a tissue culture laboratory needs separate rooms for its different activities which include;

1. **washing and drying room**
   - ✓ meant for washing and drying
   - ✓ It should have clean water, detergents, washing basin, oven, storage racks, etc

2. **Media preparation and sterilization room**
   - ✓ Meant for preparing tissue culture media and sterilization of materials
   - ✓ need to have distilled water, chemicals, autoclave, balances, magnetic/ hot plate stirrers, pH meter, meter, refrigerators, etc
3. **Aseptic transfer area**
   - ✓ meant for transferring tissue samples into prepared medium in microorganism free (aseptic) condition
   - ✓ Need to have laminar air flow cabinet (LAF) with UV light, disinfectants such as 70% ethanol
4. **Primary growth room**
   - ✓ Meant for temporary growth of cultures
   - ✓ Sometime called dark room
   - ✓ Equipped with culture racks and controlled temperature(25- or +2) & RH
5. **Culture room**
   - ✓ Room for growing cultures for a long period
   - ✓ should have culture racks, controlled light, temperature(25- or +2) & RH

During the laboratory section you should differentiate each of the tissue culture equipment and their use

2.1.2. Appropriate tissue (explant)

Explant

- The small organs or pieces of tissue that are used to start TC
- Determine initial culture in vitro and speed of multiplication
- Can be of many different kinds(stem, leaf, root, node, pollen, ovule, callus, etc).

Which explant appropriate for tissue culture? What are the factors which determine the choice of explants

- The part of the plant (the stock plant or mother plant) from which explants are obtained, depends on:
  - the kind of culture to be initiated;
  - the purpose of the proposed culture;
  - the plant species to be used.

⇨ The correct choice of explant material can have an important effect on the success of tissue culture

Criteria for Selection of plant material

- ☞ Part of plant
- ☞ Genotype
- ☞ Physiological condition
- ☞ Season
- ☞ Position on plant
- ☞ Size of explant

Desirable properties of an explant:

- Easily serializable

- Juvenile
- Responsive to culture
- Importance of stock plants

2.1.3. Growth medium

- ☞ The medium sustains the plant cells and encourages cell division.
- ☞ It can be solid or liquid
- ☞ Each plant species has particular medium requirements that must be established by trial and error

Functions

- ☞ provide H_2O, mineral nutritional needs, growth regulators
- ☞ Provide vitamins, organic compounds
- ☞ provide access to atmosphere for gas exchange
- ☞ serve as a dumping ground for plant metabolites

Composition of tissue culture medium

Note; recall the last year course related to plant nutrition requirement and come up with good understanding about them

1. Inorganic nutrients (macro-micro)

- ⇨ minerals must provide -17 essential elements
- ⇨ Macronutrients (required content in the plant - 0.1% or % per dry weight) - C, H, O, P, K, N, S, Ca, Mg
- ⇨ Micronutrients (requirement - ppm/dry weight) - Fe, Mn, Zn, Cu, B, Cl, Mo
- ⇨ Na, Se and Si are essential for some plants

2. Carbon (energy source)

- Sugar – carbon source
- sucrose
- Others – fructose,glucose
- 20 to 40 g/l, usually

3. Vitamins

- thiamine (vitamin B1) - essential as a coenzyme in the citric acid cycle
- nicotinic acid (niacin) and pyridoxine (B6)
- Inositol
- Pyridoxin
- myo-inositol - part of the B complex, in phosphate form is part of cell membranes, organelles and is not essential to growth but beneficial

4. Amino acids

- ⇒ The most common sources of organic nitrogen used in culture media are amino acid mixtures,
- ⇒ (e.g., casein hydrolysate), L-glutamine, L-asparagine, and adenine.
- ⇒ When amino acids are added alone, they can be inhibitory to cell growth.
- ⇒ Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium.
- ⇒ Supplementation of the culture medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation.
- ⇒ L-tyrosine - stimulates shoot formation

5. Solidifying agents (Support Systems)

- ☞ used to create semi-solid or solid media
  - Where in plant cultures are not submerged in the medium.
- ☞ Liquid medium can be used for many plants but it must usually be agitated to provide sufficient oxygen to the tissue.
- ☞ The common solidifying agent

- Agar (from seaweed)
- Agarose
- Gelrite (Phytagel) (from bacteria)
- Mixtures (Phytagar)
- Mechanical (bridges, rafts)
- Sand

When and why we use solid and liquid media? Do know any local material which can be used as solidifying agents?

2.1.4. Aseptic (sterile) conditions

- ➢ Elimination of microbial contaminants
- ➢ Methods of Aseptic culture(sterilization

1. Surface contaminants - principally microbial saprophytes
   - that are eliminated by **surface disinfestations**
2. Internal contaminants - principally pathogens that are
   - eliminated by **thermotherapy** (35-40 C) and culture of explants free of organisms or by antibiotics

- ☞ Maintenance of asepsis (free from microorganisms) - during
  - excision and culture - procedures are carried out in sterile laminar flow positive pressure hoods (0.3 μm filters)

Note; read other source(books or articles) and understand well the method of aseptic transfer techniques

Common Plant Tissue Disinfectants

Agent	Conc. Of active ingredient	Phytoxicity	Time(min)
Na hypochlorite(laundry bleach)	0.25-1%	Moderate	5-20
Ca hypochlorite	9-10%	Moderate	5-20
H_2H_2	3-10%	High	5-20
Alcohol (ethanol/isopropanol)	70%	High	<30 sec

These disinfectants can be used in combination and the effectiveness of these solutions is enhanced by using a wetting agent such as a detergent.

2.1.5. Growth regulators

Note; recall the last year course (plant physiology and biochemistry) related to plant hormones and come up with good understanding about them.

Growth regulatory

- ☞ **Also called Plant hormones**, **phytohormones**, growth factors or growth hormones, plant growth substances'
- ✓ The word hormone is derived from Greek, meaning *set in motion*.
- ☞ are chemicals signal that regulate plant growth and development
- ✓ regulate cellular processes
- ✓ determine the formation of flowers, stems, leaves, the shedding of leaves, and the development and ripening of fruit.
- ✓ Shape the plant, affecting seed growth, time of flowering, the sex of flowers, senescence of leaves, and fruits.
- ✓ They affect which tissues grow upward and which grow downward, leaf formation and stem growth, fruit development and ripening, plant longevity, and even plant death.
- ⇨ vital to plant growth, and, lacking them, plants would be mostly a mass of undifferentiated cells occurrence

How they regulate growth and development?

- ⇨ Triggers/control enzyme activities
- ⇨ affect gene expression and transcription levels,
- ⇨ affect cellular division and differentiation,
- ⇨ affect growth and development

It is known that applied plant growth regulators elicit specific mRNA molecules (Christianson and Warnick, 1988). This is evidence of specific genes being expressed in response to the exogenous plant growth substances.

Basic types of hormone

1. Auxins
2. Cytokinins
3. Gibberellins
4. Ethylene
5. Abscisic Acid (ABA)

The most commonly used in TC; Auxins, cytokinins

- ⇨ Affect Plant Differentiation:
- Auxin: Stimulates Root Development
- Cytokinin: Stimulates Shoot Development
- ⇨ *Generally, the ratio of these two* hormones can determine plant development:
  - ↑ Auxin ↓Cytokinin = Root Development
  - ↑ Cytokinin ↓Auxin = Shoot Development
  - Auxin = Cytokinin = Callus Development

2.2. General procedure

There is specific procedure for different type tissue culture and for different plants even for different varieties, the more general procedure are**;**

1. **Cleaning glassware**
2. **cleaning the lab**
3. **Media preparation**
4. **Explant Establishment in culture medium**
5. **Multiplication and rooting**
6. **Acclimatization (greenhouse and field establishment)**

1. **Cleaning glassware**
   - ✍ washing in (hot (70°C+)) for very contaminated one) water with commercial or lab detergents (for 5-10 min),
   - ✍ Finally rinsing with distilled water (for 5 min). Why?
   - ✍ Air dried or dried in a drying oven.
   - ✍ Storing it in a closed cabinet.
2. **cleaning the lab**
   - ✍ well organize the materials and remove all the material that are not required for the experiment
   - ✍ wash the floor, door, window of the lab with berekina or other detergents
   - ✍ disinfect by alcohol(it not be necessary)
   - ✍ Fumigation; Formaldehyde Treatment of Tissue Culture Hoods
     a. Set 2-3 Petri dish in each room(washing, transfer, culture)
     b. Put piece of sponge and add the formaldehyde so that it can vent outside.
     c. Burn it and Leave overnight and then switch on hood for at least 1 hour prior to use.
3. **Media preparation**
   - ✍ Preparation of stock solution(mass media preparation)
   - ✍ Preparation of media from stock solution
   - ✍ The prepared media depend on the types of culture, the objective of the culture, etc

   Note; In laboratory section you should understand each the step for preparation of stock solution and media preparation

4. **Explant Establishment in culture medium (practical section)**
   1. Selection of explant
   - Selection of the plant tissue (explant) from a healthy vigorous 'mother plant' - this is often the apical bud, but can be other tissue
   2. Cut into piece to get required size
   3. Sterilization
   - This tissue must be sterilized to remove microbial contaminants(section)
   4. Aseptic transfer of Culture to prepared medium (LAF)
5. **Multiplication and rooting**
   - ➢ If successful, meristem culture, shoot culture and node culture can ultimately result in the growth of small shoots.
   - ➢ With appropriate treatments, these original shoots can either be
   - ✓ rooted to produce small plants or 'plantlets', or
   - ✓ their axillary buds can be induced to grow to form a cluster of shoots.
   - ➢ Plants are propagated by dividing and reculturing the shoot clusters, or by growing individual shoots for subdivision.
   - ➢ At a chosen stage, individual shoots or shoot clusters are rooted.

   Frequent subculturing
   - to ensure adequate nutrition and to avoid the build up of waste metabolites
6. **Acclimatization (greenhouse and field establishment)**

   - ✍ The rooted shoots are potted up (deflasked) and 'hardened off' by gradually decreasing the humidity
   - ✍ This is necessary as
     – many young tissue culture plants have no waxy cuticle to prevent water loss

- a very important step in tissue culture since it is possible to lose plants if they are not properly hardened-off

❖ Have two or three phases

- Greenhouse establishment

✓ Under partial shade

✓ At normal greenhouse condition

- Field establishment

2.3. Application

The major application of tissue culture are

1. propagation
2. Germplasm preservation
3. Haploid & Dihaploid Production
4. Industrial Products(secondary metabolize)
5. Creation of variation & mutation selection
6. Transformation
7. Artificial seeds production

2.3.1. Propagation

What is propagation? Can you mention different methods of plant propagation?

Why plant propagated in tissue culture?

Plant propagation by using tissue culture help to achieve the objectives which is not possible through the conventional breeding;

❖ Mass propagation to supply enough plant materials.
=> allows fast commercial propagation/multiplication of new cultivars

❖ To propagate new cultivars, rare species, and difficult to propagate plants
=>rare and endangered plants can be cloned safely

❖ to achieve the objectives which is not possible through the conventional breeding

- For example, production of pure line involves six to seven generation s of selfing. Using anther culture, it can be reduce to two generation
  - Only method of reproduction for sterile plants such as triploids (e.g., bananas)

❖ Plant cultures in approved media are easier to export than are soil-grown plants, as they are pathogen free and take up little space (most current plant export is now done in this manner)

Advantage of propagating plant by TC

- Quick plant multiplication
- year round production(no seasonal limitation)
- Potential for disease-free propagules
- Very small explant can be used (avoids material limitations)
- Taking an explant does not usually destroy the mother plant
- Maintenance of heterozgous material(true to types)
- economical in time and space

Disadvantage

☞ Protocols not optimized for all species

☞ Plants produced may not fit industry standards

☞ Relatively expensive to set up?

- Specialized equipment/facilities required
- More technical expertise required

☞ Somaclonal variation may occur
☞ It may not work for every species

2.3.2. Long-term germplasm storage

- Cloning of plant types not easily propagated by conventional methods (few offshoots/ sprouts/ seeds; date palms, ferns, nandinas)
- Propagules have enhanced growth features (multibranched character; Ficus, Syngonium)
- Germplasm preservation
- Plant 'tissue banks' can be frozen, then regenerated through tissue culture

2.3.3. Eliminate virus from infected plant selection

- Either via meristem culture or sometimes via heat treatment of cultured tissue (or combination)
- often used for potato, strawberry, banana, citrus
  Apical meristems have faster multiplication rate than pathogens, eg. Virus
  =>Pathogens often not present in apical meristems

2.3.4. Production of Haploids

Production of Haploids *In Vitro* through Anther and Microspore Culture

Agricultural applications for haploids

- Rapid generation of homozygous genotypes after chromosome doubling
- Reduce time for variety development, e.g. 10 to 6 years or less
- Homozygous recombinant line can be developed in one generation instead of after numerous backcross generations
- Selection for recessive traits in recombinant lines is more efficient since these are not masked by the effects of dominant alleles
- Most widely used issue culture technology in plant breeding
- to create genetically engineered organisms
- One of the basic skill required for biotechnology (regeneration of transgenics)
- A more recent advance is the use of plant and animal tissue culture along with genetic modification using viral and bacterial vectors and gene guns to create genetically engineered organisms
- To create Somaclonal variation & mutation selection

Processes Leading to Production of Haploid Plants

1. Androgenesis – haploid plant derived from male gamete, most common method *in vitro*
2. Parthenogenesis - from unfertilized egg
3. Chromosome elimination - chromosome elimination in somatic cells, most common method used with plant breeding

2.4. Factors Affecting Plant Tissue Culture

1. Growth Media
- Minerals, Growth factors, Carbon source, Hormones
2. Environmental Factors
- Light, Temperature, Photoperiod, Sterility, Media
3. Explant Source

3. The genetic material

LEARNING OBJECTIVES

After reading this chapter you should be able to understand

- The structure and function DNA
- Physical and physiological(replication…) properties of DNA
- The concept of gene and gene regulation
- specificity and modality of gene expression

3.1. Discovery of DNA

What are the requirements to fulfill as a genetic material?

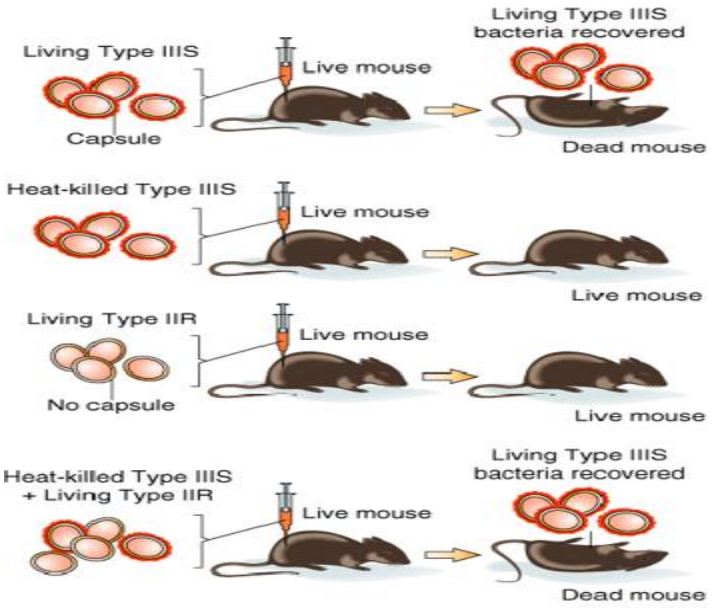
1. The genotype function or replication:
   - The genetic material must be capable of storing genetic information and transmitting this information faithfully from parents to progeny, generation after generation.
2. The phenotype function or gene expression
   - The genetic material must control the development of phenotype of the organism, be it a virus, a bacterium, a plant or animal.
   - That is, the genetic material must dictate the growth and differentiation of the organism from single celled zygote to the mature adult.

From the common macromolecules (protein, DNA, RNA, carbohydrate) in living thing which one is carries genetic information.

- For long time protein is consider as a genetic material.
- During the 1940s and early 1950s, several elegant experiments were carried out that clearly shows that **nucleic acids** (DNA, RNA) is genetic material rather than protein.
- More specifically these experiment shows that DNA is genetic material for all living organism except for RNA viruses.

The contributor for the discovery of DNA

- Mendel (1866) stated that physical traits are inherited as "particles"
- Friedrich Miescher (1869); **Isolates "nuclein"** from nucleus
- 1900: **rediscovery of Mendel's work** by Robert Correns, Hugo de Vries, and Erich von Tschermak
- Walter Sutton (early 1900s); Suggested chromosomes held hereditary factors
- 1910: Thomas Hunt Morgan proves that **genes are located on the chromosomes** (using Drosophila).
- In 1928, Griffith demonstrates the **"transformation"** of non-pathogenic bacteria to pathogenic bacteria



IIIs-smooth, virulent, has capsule

IIIR-rough colonies, non-virulent, no capsule

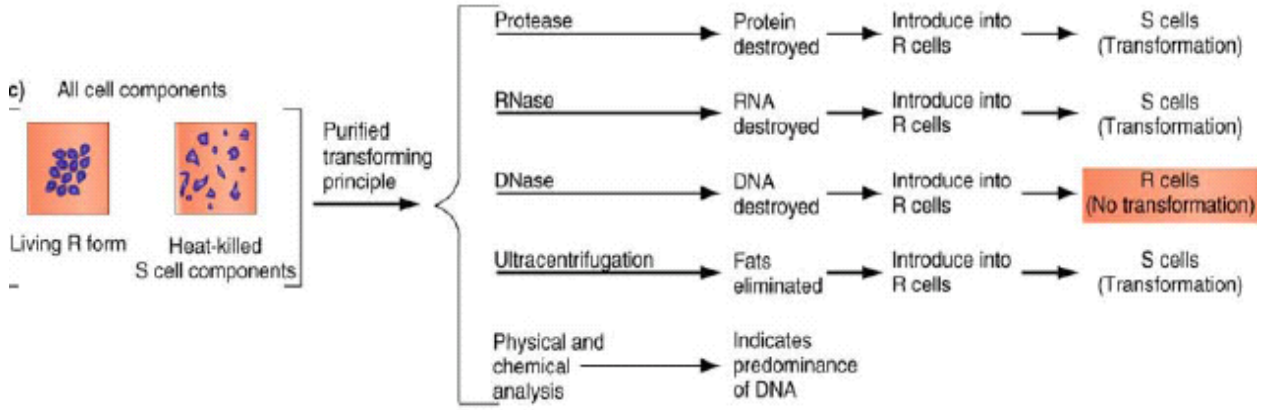
Fig. Griffith's exprement. Something in dead IIIs cells can convert IIR cells to virulent IIIs cells(transformation)

Proof that the "Transforming Principle" is DNA

- In 1944, Avery, MacLeod and McCarty gave evidence that DNA is the genetic material
- George Beadle & Edward Tatum (1950s); One-gene-one-enzyme theory, Enzyme production under control of genes
- Alfred Hershey & Martha Chase (1952); Building of Avery's work, confirmed DNA as hereditary material
- 1966: Marshall Nirenberg solves the genetic code, showing that 3 DNA bases code for one amino acid.

Alfred Hershey & Martha confirmed through the experiments that "transforming particle is DNA".

- In a highly purified DNA from Type IIIS cells was treated with:
  1. Deoxyribonuclease (DNase)
  2. Ribonuclease (RNase)
  3. Protease.



Function of DNA

- the chemical of life
- Contains all the information required to build the cells and tissues of an organism.
- carries along its length a series of coded chemicals called genes

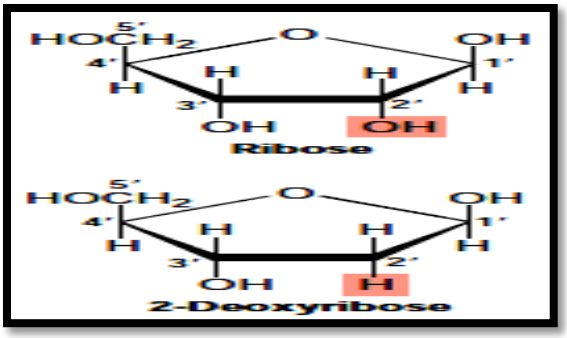
Generelly DNA serves the following functions

- It is the genetic material in all prokaryotes and eukaryotes.
- It is capable of replication by which it can be faithfully passed on to successive generations.
- It is involved in the synthesis of RNA.
- It provides the code for protein biosynthesis.
- It is involved in mutations and genetic recombinations, which bring about variations.

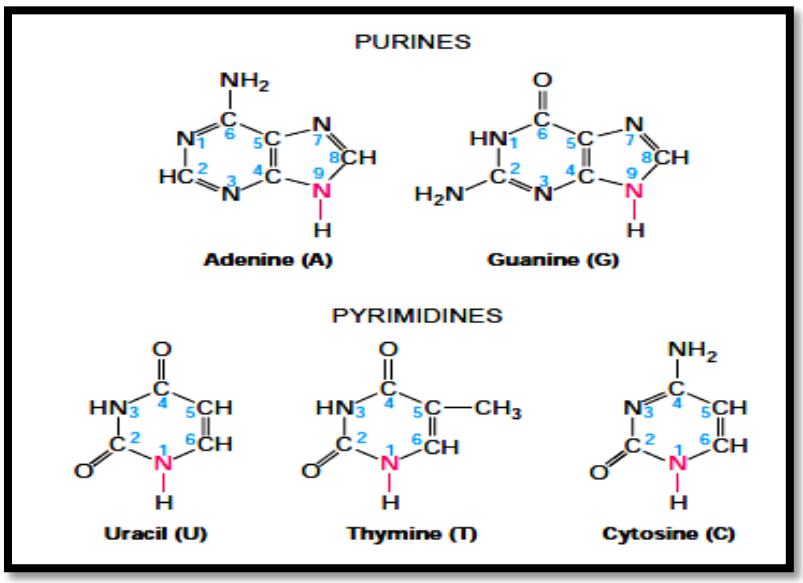
3.2. Structure of DNA

☞ Nucleic acids first called "nuclein" because they were isolated from cell nuclei by F. Miescher in 1869

- In DNA, the sugar is 2-deoxyribose (thus the name deoxyribonucleic acid)
- In RNA, the sugar is ribose (thus ribonucleic acid). Missing the -OH group at the 2'-position



- ☞ The monomer for all nucleic acids is the nucleotide
- ☞ Each nucleotide is composed of
  1. Phosphate group
  2. five – carbon sugar (or Pentose), and
  3. cyclic nitrogen containing compound called a base.
     Adenine, Guanine, Thymine and Cytosine



The Watson and Crick DNA Double helix

- The correct structure of DNA was first deduced by J.D. Watson and F.H.C.Crick in 1953.
- Their double helix model of DNA structure was based on two major kind of evidence.

1. Chargaff's rule
2. X – ray diffraction patterns.

Chargaff's rule

- The composition of DNA from many different organisms was analyzed by E.Chargaff and his colleagues.
- It was observed that concentration of thymine was always equal to the concentration of adenine (A = T)

- And the concentration of cytosine was equal to the concentration of guanine (G = C).
- This strongly suggest that thymine and adenine as well as cytosine and guanine were present in DNA with fixed interrelationship.
- Also the total concentration of purines (A +G) always equal to the total concentration of pyrimidine (T +C). However, the (T+ A)/ (G+C) ratio was found to vary widely in DNAs of different species.

TABLE 6.1 Chargaff's Data on Nucleotide Base Composition in the DNA of Various Organisms

	Percentage of Base in DNA				Ratios	
Organism	*A*	*T*	*G*	*C*	*A:T*	*G:C*
Staphylococcus aermentans	12.8	12.9	36.9	37.5	0.99	0.99
Escherichia coli	26.0	23.9	24.9	25.2	1.09	0.99
Yeast	31.3	32.9	18.7	17.1	0.95	1.09
*Caenorhabditis elegans**	31.2	29.1	19.3	20.5	1.07	0.96
*Arabadopsis thaliana**	29.1	29.7	20.5	20.7	0.98	0.99
Drosophila melanogaster	27.3	27.6	22.5	22.5	0.99	1.00
Honeybee	34.4	33.0	16.2	16.4	1.04	0.99
Mus musculus (mouse)	29.2	29.4	21.7	19.7	0.99	1.10
Human (liver)	30.7	31.2	19.3	18.8	0.98	1.03

X ray diffraction

- When X rays are focused through isolated macromolecules or crystals of purified molecules, the X ray are deflected by the atom of the molecules in specific patterns called diffraction patterns.
- It provides the information about the organization of the components of the molecules.
- Watson and Crick had X ray crystallographic data on DNA structure from the studies of Wilkins and Franklin and their coworkers.
- These data indicated that DNA was a highly ordered, multiple stranded structure with repeating sub structures spaced every 3.4 A^o (1 Angstrom $= 10^{-10}$ m)

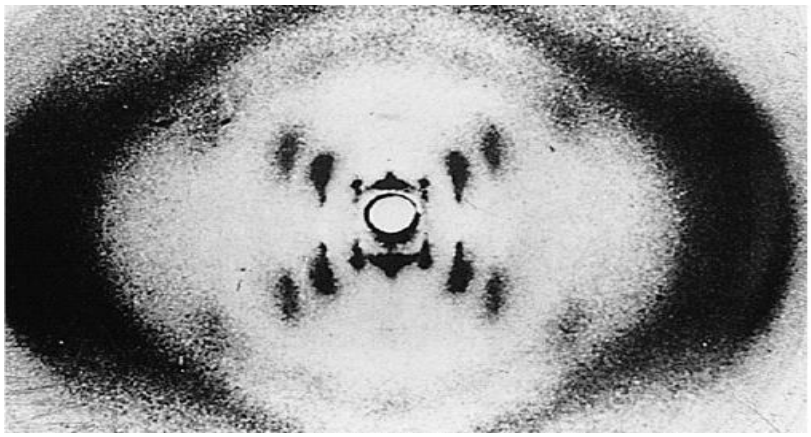


Fig. X-ray diffraction patterns of DNA– Rosalind Franklin and Maurice Wilkins. The central cross shaped pattern as indicative of a helical structure. The heavy dark patterns (top and bottom) indicate that the bases are stacked perpendicular to the axis of the molecule.

Double Helix

- Watson and Crick proposed that DNA exists as a double helix
  - in which two **polynucleotide chains** are coiled above one another in a spiral.
- Each polynucleotide chain called **strands**

The strands of DNA

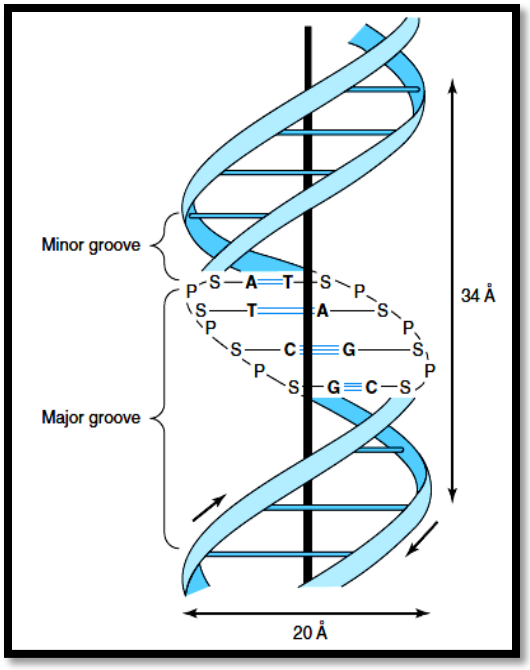
- Each strand is a very long molecule containing thousands of nucleotides
- The nucleotides Linked together by **Phosphodiester bonds**.
- They are equidistant from each other
- Held together in their helical configurations by **hydrogen bonding**
- Joined by base pairs b/n a purine & a pyrimidine
- The high degree of **stability of DNA double helices** results in part from the large number of hydrogen bonds between base pairs.
- Although each hydrogen bond by itself quite weak, since no. of hydrogen bonds are more, it can withstand
- They are

1. **anti-parallel**, i.e., oppositely oriented
   - one strand runs in 5' ⇨ 3' direction i.e., the coding or sense strand
     - Go from a 3'Carbon of one nucleotide to a 5'Carbon of the adjacent nucleotide.
   - while the other strand runs in 3' ⇨ 5' direction (5'Carbon to a 3'carbon)i.e., the template, antisense, or non-coding strand
2. **Complementary** (not identical) to each other.

⇨ It is this property, that makes DNA uniquely suited to store and transmitting the genetic information.

3. **Form 2 d/t size grooves** b/n them; Major & minor

The nucleotide in the DNA joined by phosphodister bond and the two stand are joined by H bond



The base-pairs in DNA

- ➢ **is specific**
- adenine is always paired with thymine
- guanine is always paired with cytosine
- ⇨ all base-pairs consists of one purine and one pyrimidine.
- ⇨ Once the sequence of bases in one strand of DNA double helix is known, it is possible to know the other strand sequence of base because of specific base pairing.

➢ **are stacked 34Aº apart** with 10 base-pairs per turn (360^0) of the double helix

Conformational Flexibility of DNA Molecule

Different Forms of Double-Stranded DNA

Why the structure of DNA molecules change in form?

- ☞ DNA molecules exists in various forms depending upon;
- base composition & physical conditions
- their environment (nature of chemicals it is interacting with).
- ☞ There are at least six types (A - E & Z), with **A, B & Z** forms the most common

B **form** -Watson – Crick double helix

- ☞ The vast majority form of DNA
- ☞ the DNA molecules present in the aqueous protoplasms of living cells
- ☞ form represent the 92% relative humidity.

- In fact, intracellular B-form DNA appears to have an average of 10.4 nucleotide-pairs per turn, rather than 10.

A form

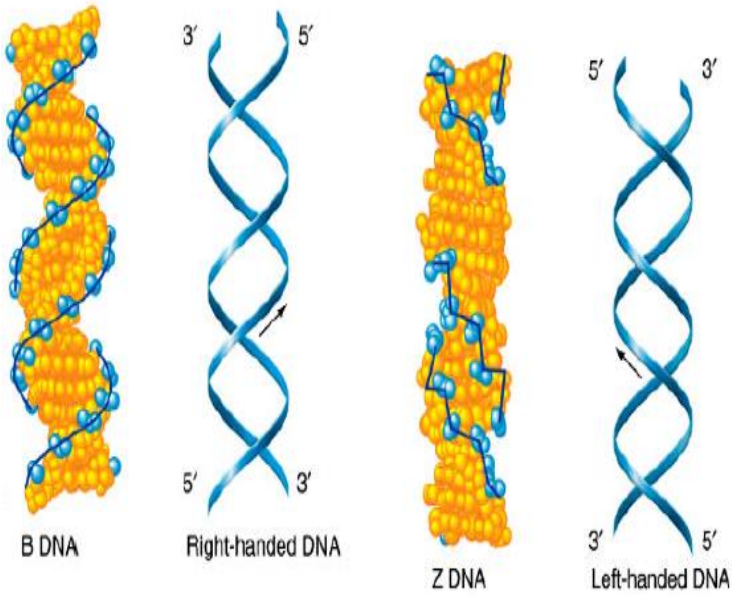
- occurs in high salt concentration or partially dehydrated conditions (75% humidity)
- doesn't exist in *vivo (living cells)*
- has 11 nucleotide-pairs per turn.

The helices of A and B form DNA are wound in a **right handed** manner.

Z-DNA

- Recently, certain DNA sequences have been shown to exist in a unique **left handed**, double helical form

Forms	Residue Per Turn	Pitch A^0
A	11	24.6
B	10	33.2
C	12	45.6



Did you know?

- **Each cell has about 2 m of DNA.**
- **The average human has 75 trillion cells.**
- **The average human has enough DNA to go from the earth to the sun more than 400 times.**
- **DNA has a diameter of only 0.000000002 m.**

- **The earth is 150 billion m or 93 million miles from the sun.**

3.3. Property of DNA

1. Replicate faithfully and transmit to offspring
2. Stores genetic information, DNA segments called genes
3. encode directions to make proteins that control cellular growth and metabolism.
4. DNA has the ability to express phenotypically and control cells (by gene expression)
5. DNA is capable of accommodating changes (mutation): DNA contains genetic changes (mutations) that are passed on to offspring.
6. DNA is stable within a living organism

3.4. DNA Replication

The process whereby new DNA is synthesized from parental DNA.

- DNA → DNA

"**Replicating Apparatus" is complex**

- DNA replication is complex.
- It is carried out by multienzyme complex, often called, replication apparatus or the replisome.
- In eukaryotes, the components of replication machinery are just beginning to be identified.
- Even in prokaryotes, DNA replication requires many different proteins

Replication fork:

- The junction between the newly separated strands and unreplicated double stranded DNA.

Leading strand

- The continuously synthesizing strand towards replication fork

Lagging strand

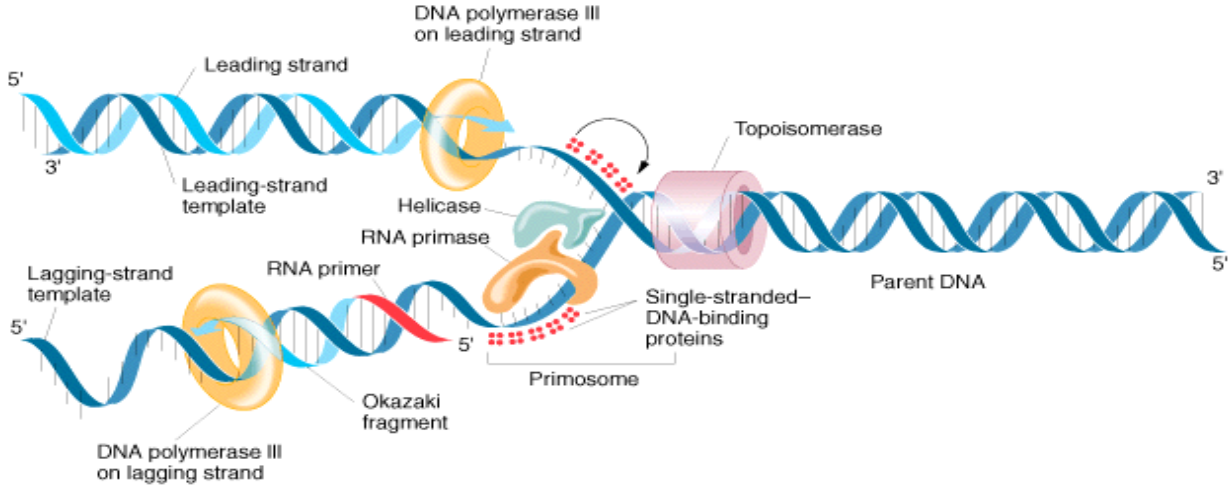
- discontinuously synthesizing strand away from the replication fork

Okazaki fragment:

- A short fragment of DNA formed on the lagging strand during replication
- It will be around 100 – 1000 bp in length.
- In eukaryotes it identified about 100-200 nucleotides length.

Processivity:

- The ability of an enzyme to catalyze many reactions before releasing its substrate



Steps and requirement of DNA replication

- ☞ To prepare DNA for replication, many proteins are involved in replication
- ☞ These proteins are required because DNA must be single-stranded before replication can proceed.
- ☞ The following are important Protein and enzyme required for DNA replication:

1. T**opoisomerase** nicks and unwinds DNA strands
2. **DNA Helicase** unwinds the molecule at replication site
3. **Single-strand binding protein** stabilized ssDNA

- ☞ Replication is 100 times faster when these proteins are attached to the single-stranded DNA

4. **Primase** initiates the replication with RNA
5. **DNA polymerase** extends the new DNA

- ☞ This enzyme recognize correct base and bind it onto the original strand by 5' end
- ☞ Keep extending the strand 5'to 3' end

6. **DNA ligase** joins all the fragments
7. **RNAse H**: To complete the DNA replication, RNA primers must be removed.

- ☛ Specifically degrade RNA that base paired with DNA. (H stands for Hybrid as RNA – DNA Hybrid)

Mechanism of DNA replication

In considering possible mechanism of DNA replication, three different hypothetical modes are apparent.

1. Semiconservative
2. Conservative
3. Dispersive

1. Semiconservative Replication of DNA

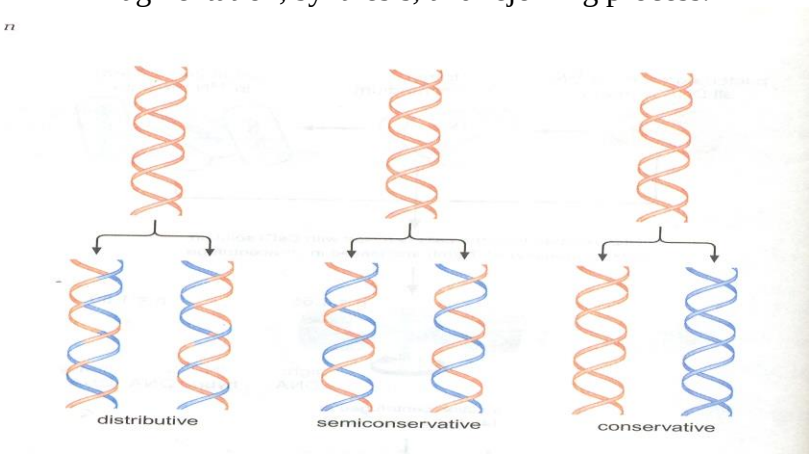
- ☞ Each parental strand could
- ⇨ direct the synthesis of a new complementary strand.
- ⇨ serve as a template for a new complementary strand.
  - Adenine for e.g., in the parent strand synthesis of Thymine in complementary strand.

2. Conservative

- ☞ Parental double helix remain intact (is totally conserved) and somehow directs the synthesis of a "progeny" double helix composed of two newly synthesized strand.

3. Dispersive

- ⇨ parental strand and progeny strand become interspersed through some kind of a fragmentation, synthesis, and rejoining process.



3.5. The Gene Concept

What is gene?

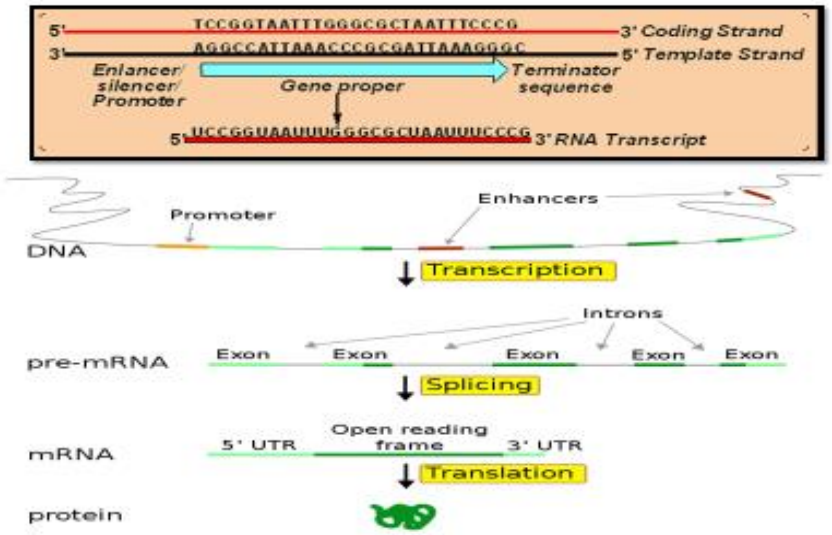
- ☞ the smallest basic physical & functional unit of inheritance encoded in the DNA of an organism
- ☞ is a specific region of a chromosome that codes for a single polypeptide (linear chain of amino acids).
- ☞ a segment of DNA (physical of heredity) that contains
- the information to produce a functional RNA (genetic code) and in turn produce a polypeptide (protein)
- segments of *DNA that* contain specific pieces of information about the traits
- ☞ are **bits of biochemical instructions** found inside the cells of every organism from bacteria to humans.

Genes

- ➢ vary massively in size ranging from 3000 bp for the shortest to 2.4 million bp;
- ➢ The human genome is estimated to contain about 35,000 – 40,000 polypeptide-coding genes
- ➢ Each gene occupies a well-defined position on a chromosome, i.e., **gene locus**

Structure of Gene

1. **Promoter**
2. **Enhance**
3. **Silencer**
4. **Exon**
5. **Intron**



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The major process of gene expression

2. Transcription-DNA to RNA
3. Spicing –RNA to mRNA
4. Translation –mRNA to protein

3.6 Gene Expression and Regulation

Basic concept, specificity and modality of gene expression

The temporal specificity of gene expression

- some specific genes in genome are expressed in order of specific time
- stage specificity in the polycellular biosomes
- The expressed genes in early developmental steps are more than in other steps in polycellular biosomes
- The expressed genes relate with biological function.

The spatial specificity of gene expression

- In the polycellular biosomes the spatial specificity of gene expression is that one or some specific genes in the genome are expressed in different systems, organs, tissues and cells in order of space.

- tissue specificity or cell specificity.
- The expressed genes relate with biological function.

Modality of gene expression

1. Constitutive gene expression -housekeeping genes

- Continual in most cells.
- The expressive products of constitutive genes are absolutely necessary in all over life process.
- are less affected by environment factors.
- are only effected by interacting between promoter and RNA polymerase.

2. Inductive and repressive

- more effected by environment factors.
- The increase of gene expression which is effected by environment factors are called **induction**, in contrast, the decrease of that are **repression**.
- The expression of induced or repressed genes are regulated by other factors, besides interaction between promoter and RNA polymerase.
- The special elements are located in the regulation region of induced or repressed genes.
- Induction and repression of gene expression correspond with each other.

Biological significance of gene expression regulation

1. acclimation
2. keep growth and proliferation
3. keep individual development
4. differentiation

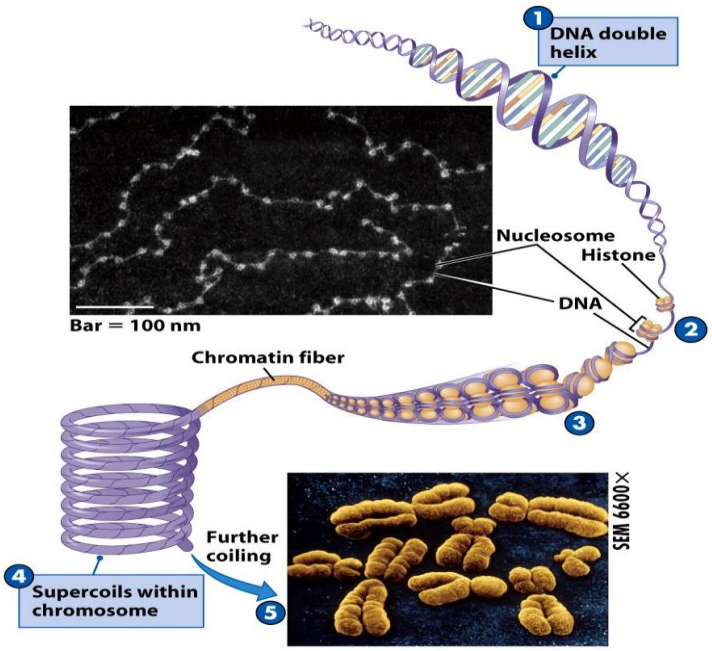
Chromosome; DNA + protein

- a single DNA molecule together with other molecules (proteins and RNA) needed to support and read the DNA.

Structural component of Chromosome

- A **gene** is a specific region of a chromosome that codes for a single polypeptide (linear chain of amino acids).
- **Centromere** (the place on the chromosome where spindle fibers are attached during cell division.)
- **telomeres** (special structures on the ends of chromosomes)
- **origins of replication** (where copying of DNA starts)
- **pseudogenes** (non-functional, mutated copies of genes)
- **transposable elements** a.k.a. transposons (intranuclear parasites)

- genes that make small RNAs and not proteins
- "junk" (?)



Repetitive DNA

- Duplications of short sequence motifs
- Interspersed -transposable elements
- Mobile genetic elements
- are present at multiple sites throughout the genome
- Classified according to the length and copy number of the basic repeat units as well as its genomic localization

1. **Minisatellites**
   - carry a common GC-rich core sequence
   - Highly polymorphic loci
2. **Microsatellites**
   - consist very short (1 to 6 bp) sequence motifs
   - characterized by a low degree of repetition at a particular locus
   - Used as a marker

Microsatellites vs minisatellites

- that microsatellites are more useful than minisatellites because:

✓ They are shorter
✓ Easier to amplify
✓ More abundant, and
✓ more evenly distributed throughout the genome

Central Dogma of molecular biology

- DNA replication; DNA synthesis
- Transcription; RNA synthesis
- Translation: protein synthesis

4. Molecular Tools and techniques

These tools and techniques

- ☞ Help to study important molecules in living organisms like
  - DNA, RNA, Proteins , Chromosome
- ☞ Helps to isolate these molecules from cell
  - To manipulate there function and structure
  - Open opportunity for their improvement and application other than their natural occurrence

How these tools and techniques are designed?

Example, A number of biological and chemical methods now give us the ability to isolate DNA molecules and to determine their base sequence. Once we have the DNA and know the sequence, many possibilities open up.

- We can identify mutations that cause disease,
- make a human vaccine in a bacterial cell, or
- alter a sequence and hence the protein it encodes.

The methods used by molecular biologists to study DNA have been developed through adaptation of the chemical reactions and biological processes that occur naturally in cells

- Many of the enzymes that copy DNA, make RNA from DNA, and synthesize proteins from an RNA template were first characterized in bacteria.

Some of molecular tools and techniques are briefly describes below

Molecular Tools

- Restriction Enzyme
- Ligase
- DNA probes
- Primer
- Polymerase chain reaction (PCR)
- Gel Electrophoresis

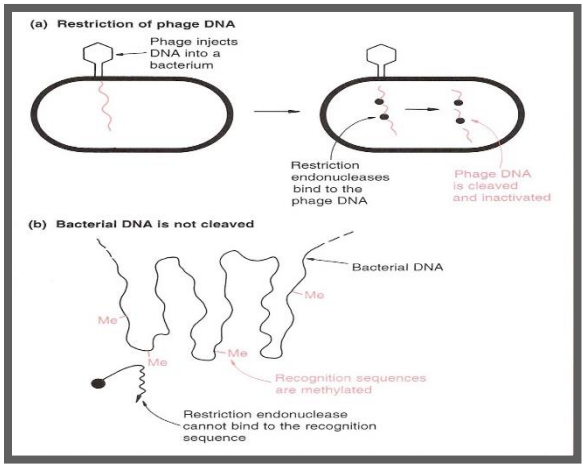
Molecular techniques

- DNA isolation
- Cutting and joining DNA molecules
- DNA cloning
- DNA sequencing
- Southern Hybridization

4.1. Molecular Tools

4.1.1. Restriction Endonuclease

- cut DNA at specific sequences
- Called as a molecular Scissors
- An enzyme that catalyzes the hydrolysis of a polynucleotide strand
- ✓ catalyzes the degradation of foreign DNA
- Originate from bacteria; produce endonucleases that digest bacteriophage DNA before infection occurs



Naming

- Roman numeral designates different enzymes from the same bacterial strain, in numerical order of discovery
- Example: EcoRI
  - E *Escherichia*
  - Co *coli*
  - R strain
  - I first enzyme discovered from *Escherichia coli* R

Step of restriction

1. Binding to foreign DNA Helix
2. Recognize short specific base sequences
   - usually 4 or 6 bases but there are some that are 5, 8, or longer
   - are **palindromes**- inverted repeats
   - Example of a palindrome:
     GAATTC
     CTTAAG
     Restriction site
3. Cutting

Restriction Sites are not evenly spaced

✓ Bgll-6sites, BamHl- sites, Sall-2sites

Blunt End vs. Sticky End

Restriction endonulceases generate two types of cut ends in double-stranded DNA.

1. **sticky ends**

– DNA molecules result from staggered cuts on each DNA strand by Some enzymes such as Bam HI, Eco RI, and Pst I

– because such fragments can associate by complementary base pairing to any other fragment of DNA generated by the same enzyme.

2. **blunt ends**

– smoothly cleave DNA fragment result from enzymes such as Sma HI

– DNA fragments produced in this way can be joined to any other blunt-ended fragment.

Parameter to select RE

A number of parameters need to be considered when choosing a particular restriction enzyme for DNA profiling experiments

- ☞ Length of the recognition sequence
- ☞ Frequency of cleavage
- ☞ Cost, and
- ☞ Sensitivity of the enzyme to cytosine methylation of the target sequence

4.1.2. Ligase

- ☞ an enzyme that **catalyzes the joining together** of two different molecules
- ☞ DNA ligase is used to complete the linking of the two DNA molecules.
- ☞ DNA ligases close nicks in the phosphodiester backbone of DNA.

4.1.3. Probe

used to identify or isolate a gene

- ☞ it is a small (25-2000 bp) length of DNA or RNA
  - Complementary to the sequence (gene) of interest

- ☞ Labeled for subsequent detection procedures

Use of probes

- ⇨ The hybridization of mRNA with its DNA gene,
- ⇨ the hybridization of chromosomal DNA with corresponding cDNA fragments, and
- ⇨ the binding of specific protein molecules to monoclonal antibodies
- ⇨ used in *northern blotting;* Southern blotting; western blotting.

Categories of probes

1. **Locus specific probes**
   - ☞ Recognize one or a few specific regions of the genomic DNA
   - ☞ resulting in easy-to-screen codominant markers
2. **Multilocus probes**
   - ☞ Are usually designed to recognize tandemly repeated DNA motifs such as mini- or microsatellites
   - ☞ These probes create complex banding patterns

4.1.4. Primer

- ❖ a short, single-stranded RNA or DNA segment
- ❖ functions as the starting point for the polymerization of nucleotides.
- ❖ Can be
  1. Specific primers
  2. random (arbitrary),
  3. universal primers

1. Specific primer

- are designed on the basis of DNA sequence information
- Can be designed based on
- unique flanking sequences complementary to know region of DNA
- Importance
- chosen for **gene isolation** or for the analysis of transferred genes in transgenic organisms
- used to analyze nuclear or organellar microsatellites(Specific primers based on unique flanking sequences)

2. Arbitrary primers

- constructed to amplify **anonymous genomic DNA** sequences under appropriate experimental conditions
- no need for initial genetic or genomic information

3. Universal primers

- designed on the basis of sequence information for **conserved parts of DNA**
- bind to conserved coding regions

4.1.5. Vectors

- ❖ Vectors are the **DNA carriers** into which "foreign" DNA or genes of interest are inserted to make a recombinant DNA
- ❖ Vectors along with this "foreign" DNA are then introduced into appropriate host cells
- ❖ They are **self-replicate**
- ❖ are divided into two categories
- – Cloning vectors – used for making millions of copies of DNA segment
- – Expression vectors – used for expression of cloned gene to produce the product

Types of cloning vectors

- ☞ Plasmids-small, circular DNA molecules that can replicate independently
- ☞ Bacteriophages –virus which infects bacteria
- ☞ Plant and animal viruses
- ☞ Transposons
- ☞ Artificial Chromosomes (BAC and YAC)
- ☞ Cosmids – artificial vectors made by combining plasmids and phages

Component of vector

- ☞ genetic markers
- ☞ transcriptional and translational signals
- ☞ Restriction (cloning) site (may & unique)
- ☞ Recognition site(multiple)or sequence

Desirable properties of vectors

- ☞ high transformation efficiency
- ☞ convenient selectable markers for transformants and recombinants
- ☞ the ability to clone reasonably large pieces of DNA

4.1.6. Polymerase Chain Reaction (PCR)

- ❖ provides an extremely sensitive means of amplifying relatively large quantities of DNA
- ❖ Can amplify one molecule of DNA into billions of copies in a few hours
- ❖ First described in 1985, Nobel Prize for Kary Mullis in 1993
- ❖ The technique was made possible by the discovery of *Taq* polymerase,

Materials, or reagents used in PCR

- ⇒ **DNA nucleotides**, the building blocks for the new DNA
- ⇒ **Template DNA**, the DNA sequence that you want to amplify
- ⇒ **Primers**, single-stranded DNAs between 20 and 50 nucleotides long (oligonucleotides) that are complementary to a short region on either side of the template DNA
- ⇒ **DNA polymerase**, a heat stable enzyme that drives, or catalyzes, the synthesis of new DNA

The cycling reactions

1. **Denaturation at around 94°C :**

- the double strand melts & open to single stranded DNA, all enzymatic reactions stop (for example the extension from a previous cycle).

2. **Annealing at around 54°C** :

- Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. If the primers exactly fit the template, the hydrogen bonds are so strong that the primer stays attached

3. **Extension at around 72°C :**

- The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

Applications of PCR

- ☞ Detection of chromosomal translocations
- ☞ Amplification across a translocation sequence

- ☞ Chromosome painting
- ☞ Detection of residual disease
- ☞ Infectious disease
- ☞ Forensics
- ☞ Detection of Loss of Suppressor Genes
- ☞ Loss of Heterozygosity (LOH)

4.1.7. Electrophoresis

- ☞ Refers to the movement of charged molecules in an electric field.
- ☞ Technique for separating the components of a mixture of charged molecules (proteins, DNAs, or RNAs) in an electric field within a gel or other support. In practice, electrophoresis employs:

Types of Electrophoresis

1. **Polyacrylamide gel electrophoresis (PAGE)** is used for the separation of smaller DNA fragments while
2. **Agarose electrophoresis** is convenient for the separation of DNA fragments ranging in size from 100 base pairs to 20 kb pairs
3. **Capillary Electrophoresis**

Gel Electrophoresis

- negative charge conferred on molecules
- gel is porous
- molecules travel through gel according to size when electrical current applied
- smaller travel faster

Reagents

- ⇨ a buffer system(Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE)).
- ⇨ A medium (paper, cellulose acetate, starch gel, **agarose gel, or polyacrylamide gel**), and
- ⇨ A source of current (i.e. electric power)
- ⇨ selective stains

Load the gel

- ➢ Gel – pure agarose (3%)
- ➢ Cover with Buffer (conducts electricity)
- Gently remove comb from gel
- ➢ Work close to electrophoresis tank
- ➢ Load wells – ***clean tip for each sample***

Run the gel

- Fit one electrode at each end of the tank
- Place in large electrophoresis tank

Factors affect the rate migration

- The strength of the electric field
- The molecular sieving action of the medium
- The size DNA fragment

Importance of gel electrophoresis:

- Gene identification, manipulation & sequencing

- Protein isolation and identification

4.2. Molecular techniques

4.2.1. Isolation of DNA from living cells

The molecular biologist need to prepare at least three distinct kinds of DNA

1. Total cell DNA
- From bacteria
- From plant
- from Animal
2. Plasmid DNA
3. Phage DNA

Is DNA isolation from d/t organism is the same or not?

- ➢ Basic steps in DNA purification are the same but with modifications
- ➢ The major modifications are needed at the cell breakage stage
- ✓ For plants – grinding frozen material are more efficient(physical method)
- ✓ Animal cells – by treating with detergent

⇒ The biochemical content of the cells from which DNA is being extracted is important consideration

The biochemical content of the cells

In most bacteria

– protein

- DNA
- RNA

Plant tissues
- Protein
- DNA
- RNA
- Carbohydrates

DNA extraction procedures, as described by different person

- microprep protocol of Fulton ***et al.*** (1995).
- Fulton et al. (1995), Rogstad (1992), and
- the Qiagen spin column extraction method (Dneasy™ Plant Mini Kit)
  - adopted to isolate genomic DNA from all samples.

Key steps for DNA Isolation

Basic steps in DNA purification are the same

1. **Lysis of the cells**

– Lysis buffer: SDS and/or 8.0M urea

– **lysozyme, EDTA for bacteria**

2. **Removal of contaminants**

– Proteins : proteinase K, phenol, chloroform extraction

– RNA- **removed by ribonuclease**

– Other macromolecules

Lysozyme

- present in egg white and secretions such as tears and saliva

- digests the polymeric compounds that give the cell wall its rigidity

EDTA

- removes Mg ions that are essential for preserving the over all structure of the cell envelope
- inhibits cellular enzymes that could degrade DNA
- Under some circumstances weakening the cell wall with lysozyme or EDTA is sufficient to cause bacterial cells to burst
- But usually a detergent such as SDS is also added
- Detergents remove lipid molecules
- ionic detergents such as SDS cause chromosomal breakage

3. Concentration of purified DNA

- Ethanol precipitation

4.2.2. Cutting and joining DNA

Restriction cutting; Method

1. Pipette in the following order:
   - Distilled water: (32-x µl), where x is volume of DNA used
   - 10x restriction buffer: 4 µl
   - Genomic DNA: x µl
   - Restriction enzyme (40 U): 4 µl
   - Total volume: 40 µl
2. Mix carefully and centrifuge in a microfuge for a few seconds to collect the ingredients at the bottom of the tube
3. Incubate for at least 3 h to overnight at the incubation temperature recommended by the supplier (37°C for most enzymes)
4. If desired, inactivate the restriction enzyme by incubating the vials at 65°C for 15 min
5. The sample can either be used directly for further processing, stored at -20°C, or ethanol-precipitated

Indication for the enzyme worked properly

- The appearance of λ-derived restriction bands of the expected sizes in the gel
- superimposed on a smear of plant DNA fragments,
- inclusion of 100 µg/ml BSA or 4 mM spermidine in the restriction buffer may help (If this was not the case,)
- Complete digestion of λ-DNA, however, is only a hint but not a definite proof for complete restriction of genomic DNA

Care for the RE

- Restriction enzymes are sensitive and expensive
  - They should not be taken out of the freezer unless actually needed
  - They should always be stored on ice or inside a cooling block
  - Fresh pipette tips should be used whenever enzymes are dispensed from the original tubes

Putting sticky ends onto blunt-ended molecule

- ☞ A common situation is where the vector molecule has sticky ends, but the DNA fragments to be cloned are blunt-ended
- ☞ three methods

6. Linkers

- It is blunt-ended, but contains a restriction site (e.g. BamHI)
- Blunt end of a molecule do not has BamHI recognition sequen

7. Adaptors

- ☞ Has one sticky end
- The idea is to ligate the blunt end of the adaptor to the blunt ends of the DNA fragment, to produce a new molecule with sticky ends
- Dimer formation of adaptors sticky end

3. homopolymer tailing

- ☞ Frequently poly(dC) tails are attached to the vector and poly(dG) to the DNA to be cloned

4.2.3. DNA Cloning: DNA Amplification

- ☞ DNA cloning is a technique for reproducing DNA fragments.
- ☞ It can be achieved by two different approaches:

2. cell based
3. Using polymerase chain reaction (PCR).

Gene Cloning vs. PCR

- ☞ Like DNA cloning, PCR results in the amplification of a region of DNA.
- ☞ Limitations of PCR

- not done in living cells, it is done in test tubes with isolated DNA
- In order to amplify an area, the sequences of the primer annealing sites must be known.
- Limited by length; regions >5 kb are difficult to amplify.
  - ⇨ DNA Cloning using vector is only way of isolating long genes or unstudied genes.

✓ PCR only amplifies one area

Requirement for Gene Cloning

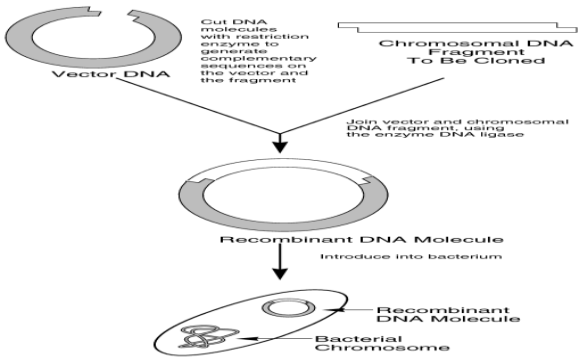
1. Vectors to transport the cloned gene into living cell(host)
2. Host to clone the gene
3. DNA to cloned
4. Enzymes; RE, ligase

Cloning process

- ⇨ Gene of interest is cut out with RE
- ⇨ Host plasmid is cut with same RE
- ⇨ Gene is inserted into plasmid and ligated with ligase
- ⇨ New plasmid inserted into bacterium (transform)

Gene Cloning

Fragment of DNA inserted into a **vector**.
Result is a **chimera** or **recombinant DNA molecule**.



Importance

- To creating many copies of the gene.
- To study gene structure and expression
- To make DNA libraries
- to prepare RNA probes from the cloned gene
- to purify large amounts of the gene product

4.2.4. In Situ Hybridization

Locating Genes in Chromosomes

- Molecules is transferred from an agar surface to a filter paper/'Membrane' like
  - paper-like matrix
  - nylon or nitrocellulose
- It also called Blotting
- The are different type
  - Southern blotting –for DNA
  - Northern blotting –for RNA
  - Western blotting –for protein

Southern blotting

- is a procedure for transferring denatured DNA from an agarose gel to a solid support filter(nitro-cellular paper) where it can be hybridized with a complementary nucleic acid probe
- The DNA is separated by size so that specific fragments can be identified

Procedure

1. Restriction digest to make different sized fragments
2. Agarose gel electrophoresis to separate by size
3. Denaturation to permit binding to the filter (NaOH)
   - Since only single strands bind to the filter.
4. Transfer to filter paper (capillary flow)
5. Hybridization to probe
6. Visualization of probe

Goals of Southern Hybridization

- Used to detect molecules in agarose gel by hybridizing with the probe
- Identify/separate DNA sequence (gene) of interest
  - To separate a particular gen of interest
- DNA fingerprinting
  - RFLP

4.2.5.DNA Sequencing

Reading the blueprint of life

- The process of determining the order of the nucleotide bases along a DNA strand.
- one of the methods of further studying cloned DNA
- DNA sequencing is important to understand the functions of genes, and the basis of inherited disorders
- DNA cloning and gene manipulation require knowledge of accurate nucleotide sequence
- Methods:
    1. Chain termination or dideoxy method(;F. Sanger)
    2. Shotgun sequence method -Used to sequence whole genomes
    3. 2^{nd} generation sequence methods, Pyrosequencing

Dideoxynucleotide method

- It is the preferred technique for determining nucleotide sequence and universally most widely used today
- Developed by Sanger in 1980
- It is an enzymatic procedure
- Commonly called Dideoxynucleotide method or chain termination method

Material required

(Sanger)

- DNA
- Rado active primer
- DNA polymerase
- dNTP's(dATP, dTTP, dGTP, dCTP)
- ddNTP's(ddATP, dTTP, ddGTP, ddCTP)

Procedure (Sanger)

1. Four reaction tubes are set up, each containing;
    a. Single stranded DNA(to sequenced)
    b. All 4 dNTPs (dATP, dCTP, dGTP, dTTP), one of them radiolabelled
    c. DNA polymerase I (sequenase)
    d. One of the 4 ddNTPs (either ddATP, ddCTP, ddGTP, or ddTTP), in much less quantity relative to the dNTPs
2. Run the reaction using DNA sequencer machine for DNA sequencing
3. Run gel electrophoresis (PAGE is used) in four lanes
4. Autoradiography using x-ray to get the DNA autoradiogram (DNA fragment bands of variable lengths on a suitable paper)

5. Markers

- ☞ A marker is a morphological trait, protein or DNA fragment that helps to distinguish one genotype from another.

Types of markers

1. Morphological markers
2. Molecular markers
   - Protein markers
   - DNA markers

Application of markers

1. Genetic diversity studies
2. Mapping
3. Marker-assisted selection in breeding

Molecular markers vs morphological markers

Characteristic	**Molecular markers**	**Morphological markers**
dominant/recessive manner	Interact	Not interact
allelic variation	Much greater	less
phenotypes and environment	independent	dependent

5.1.Morphological markers

- Are traits such as color, size, weight, height, etc., that can be scored visually and used to distinguish individuals.
- Cheap and fast

Limitation of morphological markers

- ☞ Dominance of the marker: homozygotes/heterozygotes not distinguishable
- ☞ Dependency of the environment for the experiment e.g. height of plants
- ☞ Interact epistatically, limiting the number of them that can be unequivocally scored

5.2. Molecular Markers

- A mutable site on a chromosome that is useful for cell identification and for genetic studies;
- The site of a gene of known function and known location on the chromosome.
- Reflect heritable differences in homologous DNA sequences among individuals.
- *They may be due to:*
- ✓ Base pair changes.
- ✓ Rearrangements (translocation or inversion).
- ✓ Insertions or deletions.
- ✓ Variation in the number of tandem repeats.

Advantages of Molecular Markers

- Ubiquitous
- Stably inherited
- Multiple alleles for each marker
- Detectable in all tissues, at all ages
- Long shelf life of the DNA samples

5.2.1. Protein markers

The multiple forms that enzymes take fall into two main classes according to how they are coded;

- ❖ Allozymes- enzymes coded by different alleles at one gen locus
- ❖ Isozymes- enzymes coded by alleles at more than one gene locus

Allozyme marker

- ❖ **Allozyme** polymorphisms are also used in phylogenetic and population analysis. ... Enzyme alleles can sometimes also be detected by their products, such as the blood group antigens.

Protein markers, including seed storing proteins, structural proteins, and isozymes were among the first group of molecular markers exploited for genetic diversity assessment and genetic linkage map development

- To be useful as markers, isoforms must be electrophoretically resolvable, and detectable by in-gel assay methods

Procedures

1. Grind and extract protein from appropriate tissue with buffer
2. Fractionate extract electrophoretically in starch or polyacrylamide gels (non-denaturing)
3. Detect enzyme by incubation of gel (or gel print) in a solution of a synthetic substrate that allows the enzyme to catalyze a reaction that generates a coloured product

The major limitation of protein markers are

- The availability relatively **less number** of isozyme loci in many crop species limits their utility
- ✓ Much of the genome including the most polymorphic portion of it that are less subject to evolution does code for proteins
- ✓ Proteins undergo several post-transcriptional changes and underlying DNA sequence polymorphism get masked
- Different biochemical procedures are required to visualize allelic differences for enzymes having different functions

4.2.2. DNA markers

- DNA marker are DNA fragments that differ in size due to sequence variation.
- uniquely identifiable segment of DNA.
- ✓ usually ranging in size from one to 300–400 nucleotide bases in size
- Use to study polymorphism at DNA level

Types of DNA markers

Can be categorized in several ways.

1. Based on **polymorphism**
   - polymorphic markers
   - monomorphic markers.
2. Based on **Detection of markers**
   - PCR based markers (SSR, RAPD, Etc.)
   - hybridization based markers(, RFLP)

- Combination of the above two (AFLP)

3. Based on **nature of marker**
   - dominant (RAPD, RFLP)
   - Codominant (SSR)

Importance PCR based DNA Marker

- the ability to create large numbers of markers in short periods of time
- Requires little experimental effort
- Works with nanogram amounts of DNA
- are amenable to automation
- ✓ Which is an important requisite for the high-throughput assays needed in molecular breeding programs
- ✓ any kind of primers can be chosen, depending on the purpose of the study
- – unlike traditional locus-specific, hybridization-based RFLPs
- ⇒ For example, any particular DNA sequence of interest can be amplified by a pair of specific primers

I. Restriction fragment length polymorphisms' (RFLP)

- Genetic markers resulting from the variation or change in the length of defined DNA fragments produced by digestion of the DNA sample with restriction endonucleases

Procedure

1. Different size DNA fragments are produced when digested with restriction enzymes due to variation in the enzyme's target site
2. The resulting fragments are separated electrophoretically according to size,
3. DNA is transferred by southern blotting and probed with DNA
4. Fragments matching the probe DNA are visualized by autoradiography techniques,
5. RFLP detection relies on the possibility of comparing band profiles generated

Advantages of RFLP

- Highly robust and repeatable methodology
- Codominantly inherited and can estimate heterozygosity
- No sequence information required
- Well suitable for constructing genetic linkage maps
- Locus-specific markers, which allow synteny studies
- It has high discriminatory power-both at the species and/or population levels
- Simplicity-given the availability of suitable probes, the technique can readily be applied to any species

Disadvantage of RFLPs

- Large amount of DNA is required
- Automation not possible
- Low level of polymorphism in some species
- Few loci detected per assay
- Need a suitable probe library
- Time consuming, especially with single-copy probes
- Costly
- Moderately demanding technically

II. Random amplified polymorphic DNA (RAPD)

- uses a short random primer (usually 10 bases)

⇒ Not need of genetic information

⇒ No target DNA is needed

⇒ amplify anonymous stretches of DNA

- It is dominant Marker
- When the primer attaches to two points, the region between these attachment points will be amplified

  It can detect several loci simultaneously usually resulting in presence or absence polymorphism

Advantages

- High numbers of fragments
- Technically simple
- Arbitrary primers are easily purchased, with no need for initial genetic or genomic information
- Only tiny quantities of target DNA are required
- Unit costs per assay or sample are low

Disadvantages

- Dominant (not possible to know heterozygosity)
- Lack of priori knowledge on the identity of the amplification products
- Problems with reproducibility (repeatability)

III. single sequence repeats (SSR).

- Are short tandem repeats (2-5 bases), that repeats multiple times and linkage by a unique DNA sequence.
- Also called Microsatellites
- Use specific primer

✓ designed that are complementary to the unique DNA flanking the repeated sequence

- Differences in fragment size (polymorphism) is due to differences in length (no of repeats) of the amplified product.
- to be used as markers, their location in the genome of interest must first be identified.

  Procedure

Advantages

- Require very little and not necessarily high quality DNA
- Highly polymorphic
- Evenly distributed throughout the genome
- Can be easily automated
- Good analytical resolution and high reproducibility
- Very suitable marker for marker-assisted breeding

Disadvantage

- complex discovery procedure
- High initial development cost

Desirable properties of molecular markers

1. Moderately to highly polymorphic
2. Codominant inheritance
3. Unambiguous assignment of alleles
4. Frequent occurrence in the genome

5.Even distribution throughout the genome
6.Selectively neutral behavior
7.Easy access, Easy and fast assay
8.High reproducibility
9.Easy exchange of data between laboratories
10. Low cost for marker development

Polymorphic Markers

- are those that show sequence variation among individuals.
- specific sites in the genome where the DNA base sequence tends to differ in unrelated individuals.

✓ Produce d/t types of polymorphic markers

Source of polymorphism

Polymorphisms may arise from several types of sequence variations

❖ **RFLP**

✓ Point mutations within the recognition sequence
✓ insertions or deletions between two recognition sites

❖ **RAPD**

- Insertion of a large piece of DNA between the primer binding sites
- Insertion or deletion of small piece of DNA
- The deletion of one of the two primer annealing sites
- A nucleotide substitution within one or both primer target sites

❖ **SSR**

✓ variable number of repeat units –e.g.CA)n

Nature of Marker

1. Dominancy
2. Codomintant

1. Dominancy markers

- fragments are inherited as dominant markers i.e., they are either present or absent

⇒ A fragment is seen in the homozygous (AA) as well as the heterozygous (Aa) situation, both heterozygotes & homozygous has one band

⇒ not possible to know heterozygosity

⇒ Only the absence of the fragment clearly reveals the underlying genotype (aa)

2. Codominant marker

- resulting in one or two bands depending on the homo- or heterozygous state in diploid organisms
  - One band for homozygous
  - Two band for herezygous

5.3. Application of DNA markers

1. **Genetic diversity studies**
   - Variety identification and fingerprinting
2. **Mapping of traits**
   - Mapping of qualitative traits(traits controlled by few major genes)
   - Mapping of quantitative traits (traits controlled by many genes each genes with small effects)

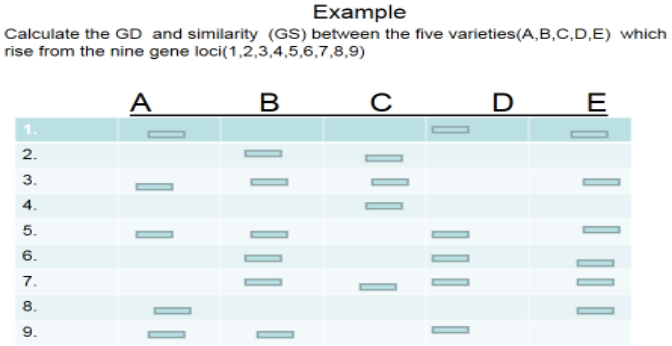
3. Marker-assisted selection

5.3.1. Genetic diversity studies

- Genetic diversity -A study undertaken to classify an individual or population compared to other individual or populations.
- The genetic distance between two samples described as the proportion of genetic elements (alleles, genes, gametes, genotypes) that the two samples do not share
- genetic distance(GD) = 1-GS(genetic similarity)
- Genetic similarity(GS) = No. of common bands
  (between two individual) total No. of bands
- Total No. of band = common bands + bands in ind.1 only + bands in ind.2 only
- Distance and similarity are inversely related

  Example

  Calculate the GD and similarity (GS) between the five varieties(A,B,C,D,E) which rise from the nine gene loci(1,2,3,4,5,6,7,8,9)



5.3.2.Mapping of qualitative traits

Steps:

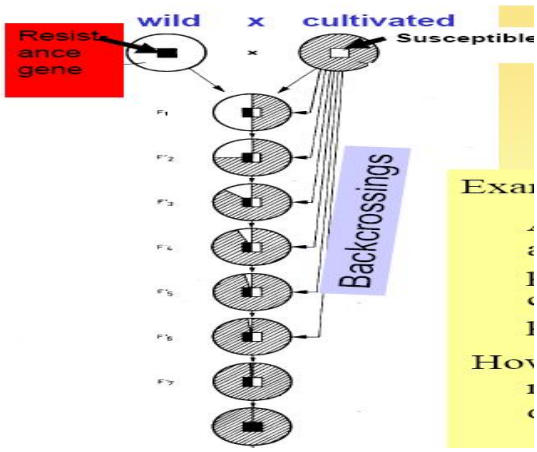
- Identify your parents; parents must be polymorphic
- Identify the markers to use
- Develop the mapping population
- Score the markers and the target phenotype in each individual of the mapping population.
- Analyze the linkage between the markers and your phenotype
- Identify several markers associated with the trait in the future

Mapping genes controlling quantitative traits

- Quantitative traits are controlling by many genes with small effects
- Genes controlling quantitative traits are referred as quantitative traits Loci(QTL)
- These traits are affected by the environment
- Variation is continuous (one cannot classify individuals into particular classes)
- Objective of QTL mapping is to identify chromosomal regions associated with our markers
- Once identify, the associated markers can be used to screen for the trait of interest in the future

5.3.3. Marker- assisted selection(MAP)

- Concept of using molecular markers particularly DNA based to detect and track presence of gene transfer in breeding programs
- MAS works on the principle of linkage where markers that are tightly linked to target genes segregate together in a non random manner.



- A breeder aims to improve the resistance of a cultivated form. Therefore, he/she performs a cross between the susceptible cultivated form with a wild form that possess the required resistance.
- However, at least 6 backcrossing steps are necessary and the resistance is difficult to detect.

Advantage of MAS

1. It saves a lot of time e.g. backcrossing
2. It requires small amount of tissue, therefore, no destructive sampling
3. Accurate identification of individuals for scoring without ambiguity: not affected by environment, phenotype (heterozygosity) stage of growth, etc
4. Individual with the desires feature can be identified before the trait is expressed, e.g. before flowering but it has addition costs

6. Genetic Engineering

Terminology

- **Recombinant DNA** refers to
  - A DNA molecule formed by combining two or more DNA molecules using *in vitro* techniques
  - a new combination of DNA molecules not found together naturally
- **Recombinant DNA Technology** (Genetic Engineering):- a technique by which new DNA (recombinant DNA) molecules are formed by cutting and joining
- **Molecular cloning**:- the multiplication of foreign DNA molecules in a host cell such as bacteria using a cloning vector that is capable of replication.
- **Genetic transformation**:- incorporation of foreign DNA by a cell and subsequent recombination of it in to the cell's genome.
- **Transgenic (genetically modified organism)**:- an organism (plant or animal, etc) containing a foreign gene (transgene) introduced by *in vitro* gene transfer techniques

Objective of the chapter

After the end of this chapter you should be able to answer the following questions

- The important of genetic engineering for agriculture
- the activities of plant genetic engineering in Ethiopia

- Limitation of genetic engineering
- Techniques of genetic engineering

Purpose of genetic engineering

Recombinant DNA molecules are produced with one of the following three objectives.

- ❖ To obtain large number of copies of specific DNA fragments:
- ❖ To produce large quantities of the protein produced by gene(expression), or
- ❖ To integrate a gen into the chromosome of a target organism

Plant breeding vs genetic engineering

- Plant breeding includes two basic steps:

1. Generation (or identification) of variation
   - ✓ Collection from wild or farms
   - ✓ Hybridization (crossing 2 or more plants)
   - ✓ Induced mutation, induced polyploidy
2. Selection for desired characteristics

In genetic engineering, the two basic steps of plant breeding are still followed, i.e., generate variation, then selection

Achievement and limitation of the tradition plant breeding

Examples of achievements of plant breeding

- ⇨ Development of triticale: an artificial cross between wheat(Triticum) and rye(secale).
- ⇨ Development of hybrid maize (more than 120Qt/ha)
- ⇨ But the world population is increasing very fast and more food is required which needs new and efficient methods of improving crops

6.1. Why genetic engineering?

- **Gene transfer is more precise** than plant breeding methods, i.e., only target genes can be transferred to the target organism which is impossible in plant breeding
- It is **faster**-establishing the trait takes only one generation compared with the many generation often needed for traditional breeding
- It is more **flexible** -genes/traits that would otherwise be unavailable in some plants or animals may be achieved using transgenic methods, by transferring genes from other organisms: practically one can transfer a gene from any organism to any other organism.

6.2. The major steps in genetic engineering

1. Identification and isolation of the desired gene
2. Constructing the recombinant gene as designed
3. Introduction of the constructed gene into a suitable host cell for cloning
4. Selection of the transformed host cells, cell with the desired clone and purification of target gene
5. Introduction of cloned DNA into the plant cell
6. Selection of transformed plant cell
7. Regeneration of transgenic plants using tissue culture

1. Identification & Isolation of gene

Example: insect resistance genes from bacteria

- Isolate the DNA using a specific probe (southern hybridization) or design a gene specific primer and isolate the gene using PCR

- Do electrophoresis and cut the band (gene fragment) from the gel and purify
  Example: Bt genes
- Spores the soil bacterium bacillus thuringiensis(Bt) contain a crystalline(cry) protein
- In the insect gut, the crystal breaks down and releases a toxin that binds to and creates pores in the intestinal lining.
- A truncated cry gene used in Bt crops.

2. Constructing the gene

Common bacterial vector promoters and replication origins are not recognized by plant cells

Major component to be include in the gene:

- **Coding sequence** of target gene: specifies the protein to be produced
- **Promoter**: initiate transcription; affects when, where, and how much gene product is produced.
- **Termination sequence**: marks end of gene
- **Selectable markers**: antibiotic or herbicide resistance

3. Introduction of the constructed gene into a host cell for cloning

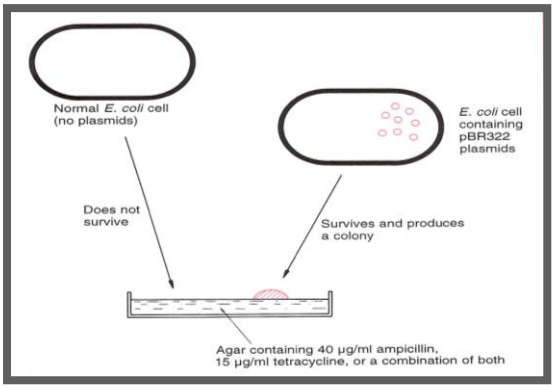
- Digest (cut) the vector DNA (eg. Plasmid) and the target DNA (gene) with the same restriction enzyme
- Mix together the vector DNA with the plant DNA using DNA ligase to ligate (join) the DNA molecules together.
- Transfer these recombinant DNA molecules (vector plus target gene) into the host bacterial e.g. E. coli to multiply (clone)
- To facilitate the entry plasmids into bacterial cells by making them porous(competent), they are treated with high/low temperature followed by $CaCl_2$

4. Selection for transformed cells

. Achieved by using selectable markers (antibiotic resistance)

❖ plated onto the selective medium

⇒ only those host cells that have taken up a vector are able to form colonies on an agar medium that contains antibiotic



5. Introduction of cloned DNA into the plant cell

It is called gene transfer and it is two type; vertical and horizontal

So-called horizontal gene transfers between species are well documented in bacteria, but much less so in fungi and other eukaryote organisms

There are different methods of transferring genes into plant cells. These include:

I. Agrobacterium method
II. Gene gun(biolistic gun) or particle bombardment
III. Electroporation
IV. Chemical method (calcium phosphate co-precipitation)
V. Microinjection
VI. Use of liposome

I. Agrobacterium method

- Agrobacterium tumefaciens inserts part of its DNA into cells of many plants causing tumors or galls
- The T-DNA enters the plant cell through a wound, then moves to the nucleus and becomes integrated into the plant chromosome.

II. Gene gun

- The Agrobacterium method works well mainly in legume crops but not in cereals
- So, a universal method that works for all species has been developed
- This method(gene gun) works using high pressure
  - ✓ Ballistic Gene Transfer- the use of tiny DNA-coated projectiles as carriers. It is important to transport DNA through the walls of intended recipient cells.
  - ✓ Projectiles are often known as micro projectiles
  - ✓ Ballaistic transformation is done by using a 'gene gun' the gene gun has been useful in creating agricultural crops.

III. Electroporation

- In a tube, plant cells are mixed with the DNA to be transferred, and placed in a small chamber with electrodes connected to a power supply.
- For a brief period (microseconds) electric pulse (about 400 volts) is discharged across the electrodes, transiently opening holes in cell membranes.
- DNA enters the cells, which then are plated in fresh medium to regenerate.

IV. Chemical method (calcium phosphate co-precipitation)

- The DNA is mixed with a phosphate solution.
- Addition of CaCl2 from a precipitate of Ca-phosphate and DNA.
- The precipitate is added to cell growing in a perti-dish and lest on the cells for several hours, during which time many of the cells take up the precipitated DNA.
- The cell are grown for 24-48 hours, then selective media is added to select for transformed cells.
- It has high efficiency of DNA uptake but usually no true transformation.

V. Microinjection

- A small amount of DNA is injected directly into the cell nucleus using micropipette and a micromanipulator.
- One can direct DNA into the nucleus, ensuring high efficiency of transformation and provides stable transformants.
- But small numbers of cells can be microinjected at one time as it is time taking

VI. Use of liposome

- Liposomes are spheres made of synthetic members of mono-or-bi-layers of phospholipids
- DNA and lipids are suspended in ether and sonicated for 20 seconds. As a result, micelles (small particles) are formed that contain DNA.
- The liposome with their DNA are mixed with the plant cells together
- The liposome fuse spontaneously to the membrane, delivering its content the cytoplasm

6**. Selection of transformed plant cells**

To identify cells or tissues in which new genes are incorporated into plant's DNA, grow in media containing antibiotics or herbicid

7. **Regeneration of transgenic plants**

- Regeneration of transformed plant cells is by tissue culture. The presence of gene in the transformed plant is checked by the following methods.

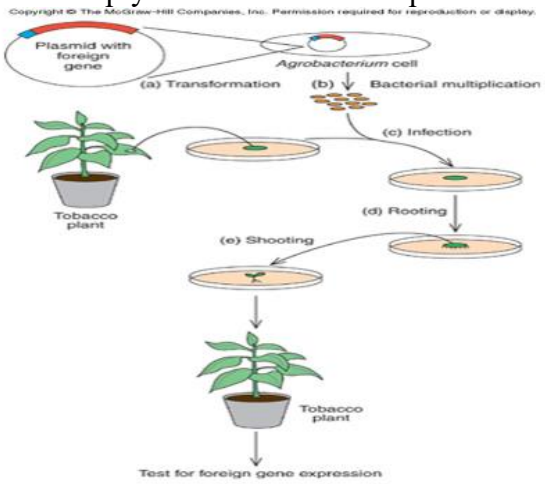
✓ Southern blot- Checking presence of a gene in the transgenic using DNA probes
✓ Northern blot- checking presence of a gene in the transgenic through its mRNA produced from the gene
✓ Western blot- checking presence of gene using its protein product

Plant tissues used for transformation

- The choice of tissue depends on the species, but some common ones are immature embryos, leaf disks, and apical meristems.
- The tissue must be capable of generating callus (undifferentiated tissue), from which the complete plant can be produced.

✓ By making a callus the number of transformed cells is increased

Plant cells can simply be mixed with a suspension of bacteria containing the transgene and vector.



How to use transformed plants?

- After proper testing of transformed and regenerated plants, they can be directly used as a variety or line.
- But often they are lines with poor agronomic or quality characteristics.
- Therefore, an improved variety must be backcrossed with them for several generations to the transformed plant.

6.3. The Applications of Genetic Engineering

- Production of protein from cloned genes
  - Bacteria cell
  - animal cell
  - farming(form living cell)
  - Plant cell
- Gene cloning and DNA analysis in medicine
  - Production of recombinant pharmaceuticals (insulin)
  - Identification of genes responsible for human diseases
  - Genetic fingerprinting by hybridization probing
  - Gene therapy for inherited diseases

Application of genetic engineering in plants

- Insect resistant plants- Bt crops
- virus resistance plant By
- "immunizing" plants- Coat protein
- expressing viral proteins in the plant cells- antiviral plant proteins
- Herbicide resistant plants- glyphosate resistance plants
- Fungus- and bacterium-resistant plants
  - pathogenesis-related (PR) proteins(e.g. chitinase)
- Development of stress-and senescence-tolerant plants
  - Oxidative stress
  - Salt stress-osmoprotectants
- Fruit ripening
  - by interfering with the expression of one or more of the genes involving in ripening(cellulase and polygalacturonase), the ripening process might be delayed
- Uniquely colored flowers can be developed by manipulating the genes for enzymes in the anthocyanin biosynthesis pathway
- to improve the nutritional quality
- , by modification of the amino acid content of some of their seed storage
- The taste of fruits and vegetables by the introduction of

6.4. Genetically Modified Plants (GMP) and Biosafety

What is a GM crop?

- genetically improved by adding or eliminating a gene or genes
- from the same or a different species
- artificially inserted in its genome.
  - via marker assisted breeding - no GM or
  - directly (through genetic engineering - GM)

Purpose of genetic modification in plants

- ✓ to produce new desirable characteristics
  - Resistance to diseases, insect. H
  - Tolerance
  - High yielding
  - Good quality
  - Early maturing

- etc
- ✓ To eliminate undesirable characteristics
- etc
- ✓ Toxicity, allergic
- ✓ The main reason for uses of GM crops is to feed a very increasing population of the world

6.4.1. Production status GM crops

- Not all aspect of GM crop discussed here:
- many are still out of reach for the moment,
- some are subject to fundamental research,
- some are at experimental stage and
- some have already been commercialized

Global status of commercialized GM crops

- China(1990s) –the first country
- USA(1994)- The first approval for commercial sale
- 1996- the first year of commercialization
- Adoption rate of GM ↑ through the 18th consecutive year(1996-2013)
- This is due to consistent and substantial
- economic,
- environmental and
- welfare benefits offered by biotech crops

Global biotech crop hectarage has increased from 1.7 million hectares in 1996 to over 175 million hectares in 2013.

1. Adoption By country

in 2013

- United States continued to be the lead country with 70.1 million hectares, with 90 percent adoption across all crops.
- Brazil ranked second for the fifth consecutive year, increasing its hectarage of biotech crops more than any other country – an impressive record increase of 3.7 million hectares or 10 percent from 2012.
- Argentina retained its third place with 24.4 million hectares.
- India, which displaced Canada for the fourth place, had a record 11 million hectares of biotech cotton with an adoption rate of 95 percent.
- Canada was fifth at 10.8 million hectares with decreased plantings of canola but maintained a high adoption rate of 96 percent.

- For the first time the top ten countries each grew more than 1 million hectares;
- 17 biotech mega countries (grown 50,000 or more ha)
- Brazil increased its hectarage of biotech crops, more than any other country in the world,
- Burkina Faso is the second

Developing countries vs industrial countries

- **Developing countries**
- Of the 29 biotech crop countries in 2010, 19 were developing countries
- grew 48% of global biotech crops in 2010

- they will exceed industrial countries before 2015
- growth rates are also faster
- The leading developing countries
- China(6), India(4), Brazil(2), Argentina and South Africa**.**

2.Adoption By Crop

- Most of the GM crops are food and fibre crops such as soybean, maize, cotton, canola, alfalfa, papaya, sugar bean, Chicory, plum, potato, rice, tomato, sunflower and squash
- The four current large hectarage biotech crops (maize, soybean, cotton, and canola),
- Biotech soybean continued to be the principal biotech crop in 2010

Examples of GM Crop Currently Available in the International Market

3.Adoption by Trait

The trait used can be classified into three categories:

- **Beneficial to the farmer**
- herbicide tolerance, insect resistance, and abiotic-stress tolerance such as drought.
- **Beneficial to processors and consumers**,
- nutrient enhancement, longer post-harvest shelf life, ease of processing.
- **produce pharmaceuticals or industrial products**
- bio-fuels, higher fiber content, higher starch composition

Most of the GM crops currently available on the market are targeted toward traits of benefit to farm

Status of GM Crops in Africa

- The GM crops that are under commercial production in Africa are
- cotton (South Africa and Burkina Faso),
- maize (South Africa and Egypt) and
- soybean (South Africa) (James, 2008)
- ……various crops and traits are under research and development
- Research for development of GM crops is on-going in a number of African countries.
- The specific crops and traits of choice in each of these countries depend on the economic importance and nature of constraints in production or utilization.

6.4.2. Challenges and Opportunities

Challenges ahead in human kind

-Population in 2050 : 9 B
-Shrinking area of cultivated land
-Diminishing water resources
-Malnutrition and undernourishment
-Deterioration in soil quality
-Climate change (global warming**)**

- The Science Alliance stated that *"The two biggest issues facing the world population today are the threat of food insecurity and the possible negative implications of climate change,"* (Scientific Alliance, 2010).

The positive Impact of GM crops as an Opportunities

- GM crops already contribute to some of the major challenges facing global society, including:

- **food security and self-sufficiency,**
- **sustainability,**
- **alleviation of poverty and hunger,**
- **help in mitigating some of the challenges associated with climate change and global warming; and**
- **the potential of biotech crops for the future is enormous.**

Biotech crops benefit food security, sustainability and the environment

Between 1996 and 2012, biotech crops have made positive contributions through: decreased production costs and increased productivity (estimated at 377 million tons) valued at US $117 billion;

- Environmental benefits by
  - ✓ eliminating the need for 497 million kg (a.i.) of pesticides;
  - ✓ reduced CO2 emissions by 27 billion kg in 2012 alone (equivalent to removing 12 million cars from the road for one year);
- conserving biodiversity by saving 123 million hectares of land from being placed in agricultural production during the period 1996 to 2012;
- alleviating poverty for 16.5 million small farmers and farm families, totaling more than 65 million people

1. Economical benefits

- through the 15 year of commercialization the farm income is positive
- It is due to a combination of higher yields and lower costs
  - low management practice
  - less ploughing,
  - reduced need for inputs
  - fewer pesticide sprays
  - less labor

2. **Environmental benefits**

- Can reduce the use of broad-spectrum insecticides-Insects resistance
- Help to recover the desert area by producing drought tolerance plant
  - by optimizing the practice of no /less till through application of herbicide tolerance.
- conserving soil and moisture
- decreasing CO_2 emissions
- The first GM maize hybrids with a degree of drought tolerance are expected to be commercialized this year in the USA
- the first tropical drought tolerant GM maize is expected by 2017 for Sub Saharan Africa

3. **Health benefits**

For consumers

- **Direct benefits** can come from
  - improving the nutritional quality of food
    - as vitamin A-boosted golden rice
    - Protein- enhanced potatoes
  - reducing the presence of toxic compounds and allergens in certain foods

  - created a no-tears onion to banish culinary crying,
  - Novel caffeine-free coffee plants.
- Works like any vaccine, **Edible Vaccines**
  - Humans eat the plant
  - The body produces antibodies against pathogen protein
  - Humans are "immunized" against the pathogen
  - Examples:
    - Diarrhea
    - Hepatitis B
    - Measles

- **Indirect health benefits** can come from
  - ✓ diminished pesticide use
  - ✓ less insect or disease damage to plants
  - ✓ increased availability of affordable food, and
  - ✓ the removal of toxic compounds from soil

Challenges

1. Expected Environmental risk

Cause of risk	**Types of risk**	**Effect of risk**
the transgene itself	**Gene Flow**	Extinction risk of close relatives Depletion of genetic diversity "Genetic pollution"
The Whole Plant	the weediness or invasiveness	**the supper weed**
transgene product	non-target hazards	Affect beneficial insect
Targeted pest	resistance evolution	

the two most widely discussed cases

- possible contamination of wild populations of maize in Mexico with transgenic pollen, Quist and Chapela (2001)
- the possible killing of monarch butterfly larvae by maize pollen expressing Bt toxin incidentally deposited on milkweed *(Asclepias syriaca)* plants. (Losey et al. 1999, Jesse and Obrycki 2000)
- The conclusions reached by Quist and Chapela is have been strongly criticized (Kaplinsky et al., 2002; Metz and Futterer, 2002; Mann, 2002).

2. Expected risk on human health

❖ concern about food safety of GMO food

I. Food allergy

- Allergens and toxins naturally occur in some plants

✓ Some people are allergic to some foods and others are not

II. Genes transfer in the digestive tract from a food product into human cells or to bacteria

- the potential transfer of genes from consumed food into human cells or into micro-organisms within the body antibiotic-resistance genes

⇒ lead to the development of antibiotic-resistant strains of bacteria**.** Exmple

- in 2001 there were reports that Starlink GM corn, which was approved in the US for animal feed only, was found in some food products.

3. Information gap

4. lack of knowledge

5. Unfamiliarity with the technology

6. Lack of reliable information

7. Negative media opinion

10. fears of the unknown

11. Opposition by activists group

12. Mistrust of the technology

13. Unsafe & inefficient gene transfer methods

- delivering the DNA into the cell without regard to its ultimate intracellular location
  - nonspecific recombination process
- selectable marker genes
  - may be expressed in the commercial crop to unnecessary levels
  - If so remove
    - the recent refinement of the cre-lox system (Corneille et al., 2001) is an example

14. political Issue

- seeing them as part of globalization and privatization, as being "anti-democratic" or "meddling with evolution".
- In turn, governments often
  - lack coherent policies on GMOs,
  - have not yet developed and implemented adequate regulatory instruments and infrastructures
- As a result, there is no consensus in most countries on how biotechnology, and GM crops in particular, can address key challenges in the food and agricultural sector
- developing nations have become too dependent on GM products
  - self-sufficiency of developing nations are threatened
- politicians maximize their own welfare
- behavior to get re-elected

- companies have gained far too much control over the production of crops and foods
- trade tensions between the USA and the EU
  - Large European firms simply may not be very good at genetic engineering; moreover, allowing crops such as Monsanto's Roundup Ready® varieties would

increase the use of Monsanto's Roundup herbicide instead of competing herbicides produced by European firms

6.3.BIOSAFETY

Biological risk analysis

- Risk is an integral part of everyday life
- No activity is without risk
- In some cases inaction also entails risk
- Agriculture in any form poses risks to farmers, consumers and the environment

Is there any zero-risk technology?

- It is generally accepted by governmental regulatory agencies that it is impracticable and likely impossible to adopt a zero-risk policy
  - As with almost any scientific application or technology, biotechnology poses potential risks associated with the benefits it provides.
  - Most technologies are used in spite of the risks they create and over time, societies provide protective measures to reduce risk
  - Risks of transgenic plants, as is done for other scientific and technological developments, are determined using a risk assessment

There for the GM crops may have the following expected problem

1. in health

- genetically modified foods
- ✓ The possibility of long-term effects on health
- ✓ **Allergens** and **toxins** naturally
- ✓ the potential transfer of genes from consumed food into human cells or into micro-organisms within the body

⇒ could lead to the development of antibiotic-resistant strains of bacteria(antibiotic -resistance genes as markers)

- Direct effect in the Env't
- Horizontal gene flow
- A controversy arose about whether pollen from Bt plants could harm beneficial species (such as the monarch butterfly)
- indirect effect in the Env't
- The extensive use of herbicides and insect resistant crops could result in the emergence of resistant weeds and insects

- Scientists generally agree that genetic engineering can offer some health benefits to consumers
- Direct benefits can come from improving the nutritional quality of food and from reducing the presence of toxic compounds and allergens in certain foods
- Indirect health benefits can come from
- diminished pesticide use
- less insect or disease damage to plants
- increased availability of affordable food, and
- the removal of toxic compounds from soil
- These direct and indirect benefits need to be better documented